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The undersigned hereby certify that they
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Culture of Erythropoietic Tissue In Vitro submitted by
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THE UNIVERSITY OF ALBERTA

FURTHER STUDIES OF THE CULTURE OF ERYTHROPOIETIC

TISSUE IN VITRO

A DISSERTATION

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE.

FACULTY OF MEDICINE

DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY

by

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ABSTRACT

This thesis describes the development and
improvement of technical procedures for the preparation and
culture of hematopoietic tissue (bone marrow) in fluid medium.

A historical review and critical survey was
made of the general methods of culturing used by previous workers.
In addition a review of erythropoiesis and associated phenomena
was presented with consideration of several continuing problems.

The development of a new and relatively atraumatic
preparation of the marrow utilizing native serum was outlined and
the advantages over other techniques were discussed. The results
of culturing in various media, and the effect of such substances
as heparin, liver extract, colchicine was reported. The significance
of agitating cultures during incubation was discussed and the
possibility of refrigerated storage of marrow suspensions was
investigated.

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INTRODUCTION

This thesis concerns scientific investigations
in connection with the development of a new procedure for the
preparation and culture of bone marrow.

The project was initiated by Nelson and McLuhan
(43) in 1951, working in this laboratory. Utilizing "in-vitro"
cultures of rabbit bone marrow, their experiments were directed
at an attempt to detect an erythropoietic inhibitor in the blood
of cancer sufferers. The technical difficulties experienced in
the course of their work indicated, as did the reports of other
workers, that the need for the development of an improved method
for the preparation and culture of bone marrow precluded further
studies on the original problem.

The thesis presents an extensive review of the literature; it describes the origin and evolution of modern tissue culture methods, with emphasis on hematological studies; it examines the methods of many previous workers, with reference to such points as the source and manner of obtaining and preparing bone marrow, the equipment used, the length of time of the procedure, the culture medium, and the means of assaying growth and morphology. Since the work is so closely associated with erythropoiesis in general, a survey of the theories of red blood cell production has been made with special attention to the various contradictory theories on the maturation of the normoblast, the immediate precursor of the erythrocyte.

The experimental work consisted of an attempt to produce a method of preparing and culturing bone marrow which is short and simple, physiological and atraumatic, and utilizes inexpensive equipment. Results are presented of cultures carried out under various conditions and using marrow obtained and processed by several different techniques.

It is hoped that the development of this procedure will provide an avenue for further study of the morphology and physiology of hematopoietic tissue, in both the normal and pathological states, and lead to the investigation of the effects of many physical conditions and chemical compounds on the production of blood cells.

REVIEW OF THE LITERATURE

I. TISSUE CULTURING

A. The Origin and Development

In 1778, Claude Bernard (60), by pointing out the importance of environment, suggested the need for the development of tissue culturing methods. According to his conception, in attempting to study the manner in which living cells affect their environment and are affected by the environment, these cells must be isolated in an artificial system in which the influences of the organism as a whole are absent.

The initial steps in the science of tissue culturing were made by Roux in 1885 (60) working with organized

chick embryo tissue. Thirteen years later, Ljunjyren (60) kept human skin alive for weeks in ascitic fluid. In 1903, Jolly published a paper reporting that he had maintained hanging drop cultures of amphibian leucocytes up to one month.

The publication of Harrison's work (60) is generally considered to mark the inception of a simple technique allowing for growth and development of isolated tissue "in-vitro." He was able to maintain for some time, a bit of embryonic neural tube in hanging drops of clotted lymph from an adult animal, and moreover, observe the development of nerve axones from the primitive neurons.

The possibility of great advances in the study of cell physiology was recognized by Alexis Carrel. He quickly realized that while cells survive for a short time when cultured by the Harrison method, "they are subject to complex and obscure influences such as necrotic cells of their own type, living and dead cells of other types and a medium which deteriorates within a short time" (14). By 1925, Carrel had published over thirty papers on the subject of tissue cultures, all contributing towards the goal of pure strains of cells cultured for long periods in media of unchanging composition. In addition, many papers were written by others including Ebeling, Lewis and Lewis, Champy, and Fischer dealing with the isolation and preservation of pure strains of cells, new equipment and techniques for culturing, and methods of

ascertaining the degree of growth. A great deal of work was done
on growth promoting and inhibiting factors found in embryonic and
other tissue extracts and in serum and plasma. Many studies in
cellular morphology and differentiation were also reported. Much
of the success of tissue culturing results from the extensive
investigations on the composition of culture mediums, notably the
work of Tyrode (60) and the Geys (31).

The application of Harrison's method entered many
fields. Following Carrel's isolation of a pure strain of fibro-
blasts, other types of cells were isolated and cultured.

Experimental embryology was carried out using
tissue culturing methods. Fell and her associates (27) were able
to develop a femur from the limb bud of a five-and-one-half-day
old chick embryo. Lewis and Gregory (40) took sequence photographs
of the delivery of rabbit ova washed from the oviduct and sealed in
culture slides on a warm stage.

The study of pathological conditions by culture
methods was initiated as early as 1911 when Carrel and Burrows (8)
successfully cultivated several types of cancerous cells. Sub-
sequently, many studies were initiated concerning the morphological
and biological characteristics of tumor. Investigation into the
characteristics of leukemia have been facilitated by the use of
peripheral blood and marrow cultures (62, 32) and more recently
many papers have appeared concerning the immediate cause of perni-
cious anemia (76, 81, 38, 26).

Since virus require living cells to multiply, the utilization of tissue cultures greatly facilitates studies concerning their mode of infection and multiplication. Parker and Nye (59) in 1925 proved that virus could be maintained and in fact multiply grossly when incubated in close contact with living cells in culture. Since that time extensive work has been carried out in connection with tissue specificity and immunology and antibody formation.

In the years since 1945, interest has been high concerning the effect on cells of various types of radiation. Earle (21) published the results of exposing cells in culture to radiation in the ranges visible to the human eye. Meldolesi and Guisti (20) noted that when bone marrow cells in culture were exposed to radium radiation, the less differentiated cells were killed. From this, they rationalized that the anemia and leukopenia of radiation injury was due to affected precursors.

Tissues in vitro provide an excellent medium for the biological assay of therapeutic drugs. Repeated publications (33, 1) have appeared concerning the activity of various liver extract and Vitamin B_{12} preparations in the maturation of red blood cells. Osgood (56a) made a study of sulfonilamide and related compounds using human sternal marrow.

B. Tissue Cultures of Blood and Blood Forming Tissue

The study of blood and blood forming organs began very soon after the birth of tissue culturing. In fact, one of the first tissues used was femoral bone marrow and much of the culturing during the earliest five years was associated with the study of hematopoiesis. Up to the present, the interest in bone marrow and blood cultures has at least equalled studies with other tissues.

Carrel and Burrows (8a) in 1910 culturing explants of cat femoral bone marrow, observed that in 24 hours many nucleated and non-nucleated blood cells surrounded the explant. During the same period, Victor Emmel (22, 23, 24) was making a study of the blood of pig embryos. Following observations in plasma cultures and hanging drops, he published evidence of the cytoplasmic budding theory of erythrocyte production. Using a similar technique he studied the blood of non-mammalian vertebra and noted that there too, non-nucleated erythroid cells were formed by cytoplasmic budding.

Foot (30) used explants of bone marrow in plasma hanging drops, to study the origin and development of blood cells. He described in detail the appearance and activity of various cell types in the cultures. He was able to store marrow for 24 hours in an ice box and obtain vigorous growth upon incubation. He noted that fibroblast-like cells often completely replaced other elements.
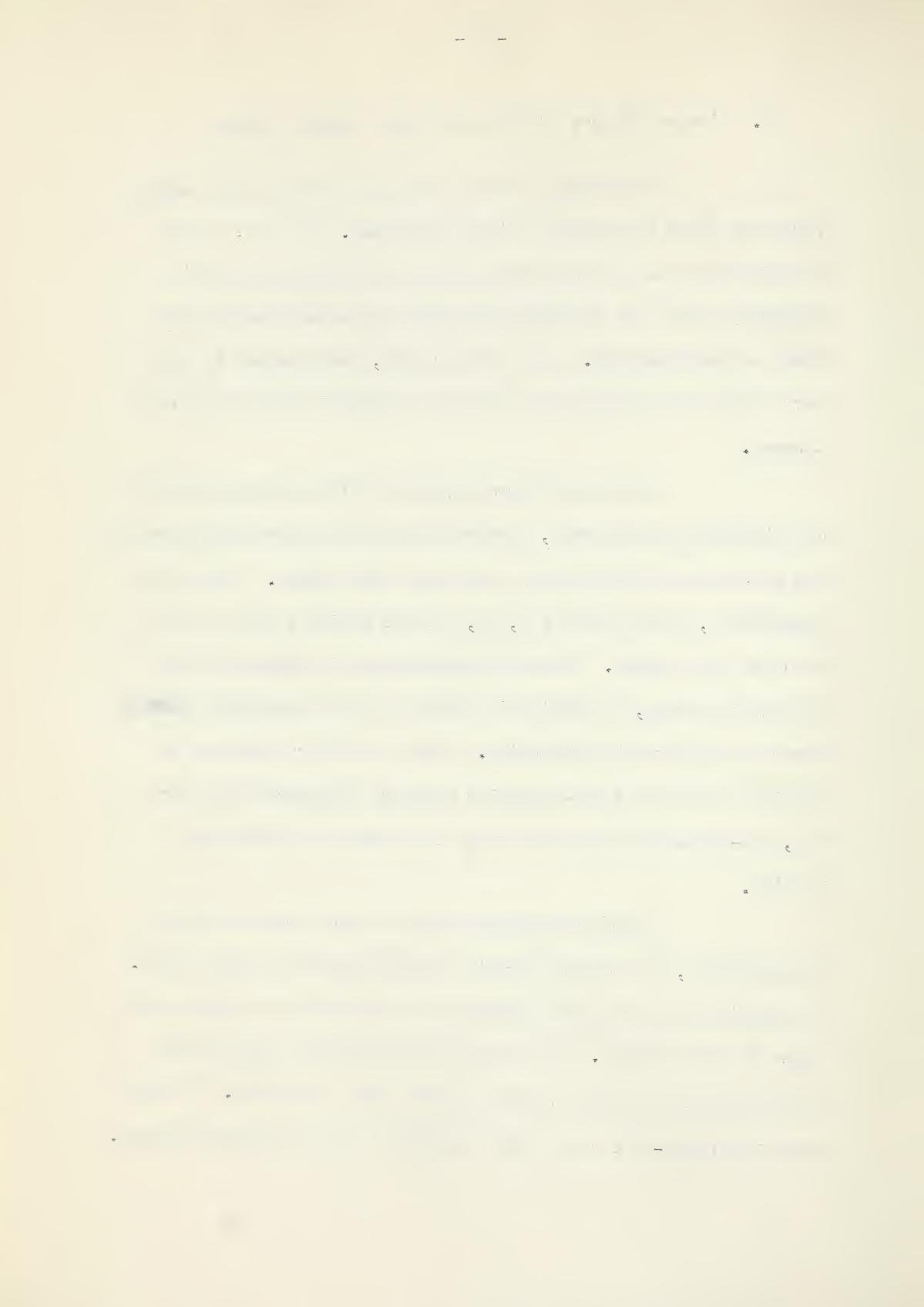
Rhoda Erdmann (25) in 1917 published a paper on her extensive work of the previous several years, in which she used a technique similar to that of Foot in studying the morphology of cells seen in culture. At varying intervals after the initiation of culture, the original explant of bone marrow was removed from the plasma and the course of the cells which had migrated into the medium was followed throughout the duration of the culture. She described the culture as consisting of two periods. During the first days, "the regressive period," all the cells which were incompatible with the conditions of the culture degenerated. Very little multiplication took place. The culture period, following the third day, was designated as the "progressive" or "generative" period. Here cells adapted themselves to the conditions of the culture and began to change their morphology and proliferate.

Carrel and Ebeling published several papers (10, 11) in which they reported the culture, in a solid coagulum, of cells of the "buffy coat" of peripheral blood. They studied the morphology of active mononuclear cells and the action thereon of homologous serum* and tissue extract. They noted that lymphocytes can live in serum which does not support fibroblast growth, and that the active lymphocytes actually produce a growth principle necessary for fibroblast growth.

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*The terms "homologous serum" and "native serum" are interchangeable in this thesis and carry the same meaning; serum from the same animal as the tissue for culture.

Lewis (39) incubated simple hanging drops of peripheral blood to study the "part taken by white blood cells in various conditions," with stress on the morphological changes and phagocytic properties.

A great deal of work was done on the nature of leukemia and leukemic peripheral white blood cells. Awrorow and Timofejewsky (1914), Timofejewsky and Benewolenskaja (1926-1929), Hirschfeld (1927), Hirschfeld and Klee-Rawidowicz (1928), Veratti (1928), Parker and Rhoads (1928) all utilized "in-vitro" culture methods (as reviewed by Pierce (62)). Pierce (62) cultured the blood of several leukemia patients using Maximow's method. She concluded her report with the hope that tissue culturing may prove valuable in the differentiation of "blast" type leukemias.

In 1936 Osgood and Muscovitz (52) published a paper in which they proposed the culture of bone marrow in a large apparatus which provided all the functions of the lungs, kidney, and liver, allowing careful control of pH, gas pressure and composition, temperature, supply of nutrients and removal of metabolites. The method was revolutionary, especially in consideration of the fact that the human cells obtained by sternal puncture were cultured as a suspension in fluid medium. It allowed frequent sampling without interference or disruption of the culture, permitting continuous hemocytometric, morphological and metabolic studies. The original apparatus proved to be much too complex for efficient research and

was abandoned. In two papers Osgood and Brownlee (53, 54) described a simple technique for the culture of bone marrow cell suspension in small vaccine vials. This technique has been used, with various modifications by the majority of investigators who have studied bone marrow "in-vitro" since 1936. Applying the vaccine vial method, Osgood and his associates carried on extensive studies on the length of life of various white blood cells (55), sulfa compounds as therapeutic agents (56a) and the effect of roentgen radiation on marrow cells (57).

Rachmilewitz and Rosin (74, 74a, 78), culturing bits of rabbit bone marrow in small tubes of clotted plasma, investigated such fundamentals as the effect of hemoglobin and red cell stroma and various oxygen tensions on bone marrow "in-vitro."

Among the results of innumerable investigations published between 1940 and the present, the works of Claus Munk Plum, and Norris and Majnarick are especially notable. Plum became interested in erythropoiesis, particularly the maturation of the normoblast to the erythrocyte. In two papers (65, 67) he described the construction of an apparatus modified from Osgood and Muscovitz (52) for the continuous culture of bone marrow. Studies were carried out on normal and pathological marrow cultured in native and foreign serum*, liver extract and folic acid (66). Later he and others

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* "Foreign serum" refers to serum from an individual or animal other than the donator of the tissue to be cultured, but of the same species.

investigated the nature of pernicious anemia and lymphatic leukemia
(15). To facilitate uninterrupted direct observation of cells in
culture, he designed a micro culture chamber (65, 69), and was able
to observe continuously and even take a moving picture of the pro-
duction of erythrocytes by cytoplasmic budding (68, 70).

Norris and Majnarich published several papers (44 -
51) recording a series of experiments concerning the effect of
various pteridine compounds of bone marrow "in-vitro." They were
able to relate the actions of Xanthoptin and some of its deriva-
tives to growth promoting and inhibiting substances found in
normal, pregnant and cancerous serum and urine. Cancerous serum
depressed proliferation "in-vitro" and this could be countered
by addition of Xanthopterin. They isolated a compound from urine,
Vitamin B_{14}, which was millions of times more active than Xanthop-
terin.

It has been said that to culture cells merely for
the purpose of culturing cells is a waste of time, money and
effort. The application of "in-vitro" culture methods as a medium
of investigation is growing more popular as the advantages become
known. The future promises great advances in medical science via
this pathway.

II. ERYTHROPOIESIS AND ASSOCIATED PHENOMENA

A. Introduction

In the culture and study of bone marrow "in-vitro,"
it is of prime importance to understand the physiology of the
origin and maturation of the cells of the marrow, both the erythroid
and the myeloid series. However, a review of the literature on a
subject such as this is difficult for several reasons. Much
original and important work was done many years ago and the publi-
cations are only rarely available; therefore, the information must
be gleaned "second hand." The nomenclature of various authors
differs from the nomenclature in common use today; many workers
coined their own terms as needed, often for cells or structures
which already had been named at least once. Most of the work was
done by means of interrupted observations. To cite an example;
in studying the formation and maturation of the cells of the blood,
most workers made fixed sections (with the danger of artifacts)
and attempted to relate to one another, cells in different stages
of maturation, observed in many sections.

The bone marrow, the site of erythropoiesis and
leukopoiesis, is the largest organ of the body, weighing 4,000
grams, twice the weight of the liver (37). It can be subdivided
into two portions. The red marrow, occupying the lumena of the
sternum, ribs, vertebrae, the flat bones of the skull and pelvis
and the ends of the femura and humeri, is the hematopoietic or

Figure 1.   The Distribution of Marrow.

The shaded areas indicate the
location and intensity of
hematopoietic marrow.   From
"The Anemias" - a publication
by Eli Lilly & Co., Indianapolis.

active portion of the organ. This red marrow, about half of the
total in the normal adult, is the source of supply of the myeloid
and erythroid cells of the peripheral blood. The yellow marrow,
which occupies the shafts and the distal ends of the long bones,
especially near the extremeties of the appendages, consists of fat,
and represents the reserve of the organ. This fat is very labile
and in times of demand can be quickly replaced by cellular tissue
which actively contributes to the cellular component of the peripheral
blood (79). At birth, the entire marrow cavity is filled with cell-
ular tissue. Within a few years much of this is replaced with fat,
the process slowing but continuing with advanced years until death
(17).

In healthy adulthood the cellular portion of the
marrow is composed of roughly 60% myeloid cells and 20% erythroid
cells. These figures are an average of the results of many investi-
gators as reviewed by Plum (64).

As far back as 1868, Neumann first described the
production of blood cells in the bone marrow. However, accurate
detailed study in the identification and differentiation of blood
and marrow cells dates from Ehrlich's work with specific dye
substances. Since that time, there have been many persistent problems
with many suggested solutions for each. Do all the cells of the
blood stem from a common precursor, or is there more than one type?
Where in the marrow do the myeloid and erythroid cells mature and how
are they released into the circulation? What are the steps in the

maturation of the myeloid and erythroid cells? How does the normo-
blast lose its nucleus in the final stage of erythrocyte maturation?

B. The Origin of the Marrow Cells

The entire cellular component of the blood has its
origin in the mesenchyme cells of the embryonic mesodermal layer.
There is, however, much controversy over the immediate progenitor
of the recognized precursors.

As one traces back the precursors of the well
differentiated mature cells, the characteristics of differentiation
become less and less obvious until identification of the general
cell type (erythroid, myeloid) becomes, at the best, very difficult.
This, in conjunction with the lack of a method for continuous
uninterrupted study of cells from the very immature to the mature
state, is the basis of the conflicting theories on the "stem or
mother cell."

1. The Monophyletic Theory (Unitarian)

The morphological similarity between the immature
myeloid and erythroid cells has promoted many writers to theorize
that all the blood cells take their origin from a single progenator
more specific and mature than the mesenchymal cell, variously termed
the hemoblast, lymphoidocyte, polyblast, hematogenous stem cell.
This single cell type, depending upon the environment, gives rise to
all types of immature blood cells, both red and white. The many

PAPPENHEIM'S THEORY

NORMOBLAST ⟶ ERYTHROCYTE

LYMPHONORMOBLAST

LYMPHOMACROBLAST

INTRAVASCULAR

LYMPHOIDOCYTE

MACROLYMPHOCYTE   SPLENOIDOCYTE   MACROLYMPHOMYELOCYTE

EXTRAVASCULAR

LYMPHOCYTE   MONOCYTE   LEUCOBLAST-MYELOCYTE

LYMPHOCYTE   POLYMORPHONUCLEAR
of the blood   LEUCOCYTE
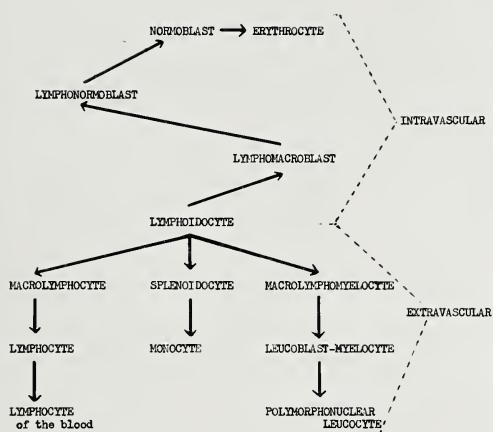
slightly modified from Sabin, Cunningham and Doan.

Figure 2. Papenheim's Theory of Blood Cell Genesis.

supporters of this theory (which varies slightly in form, depending on the author) includes such names as Virchow, Saxer, Pappenheim, Danchakoff, Maximow, Schmidt, and Herzog and Roscher (as reviewed by Cunningham et al (16).

Pappenheim theorized that the first free stage of blood cells to develop from the primitive mesenchyme is the lymphoidocyte, a form similar to the large lymphocyte. The red cell lymphoidocyte is intravascular and the white cell lymphoidocyte is extravascular. Danchakoff in a study of chick embryos noted that endothelial cells form capillaries around the common precursor, the hematoblast. The intravascular hematoblasts proliferate, some becoming small mature red cells, and some retaining their undifferentiated character, and in turn, proliferating. The extravascular hematoblasts similarly produce granulocytes. Maximow noted that the proliferation of embryonic capillary endothelial lining produced free cells which he identified as lymphocytes, from which all other blood cells arise. Many others suggested similar ideas, relating the stem cell to the mature lymphocyte. Jordan (35) described the formation from both mesothelium and endothelium of a stem cell which gives rise to erythrocytes, myelocytes and lymphocytes. Smith and Herzog and Roscher maintained that the common stem cell originated by mitoses of the endothelium and not from the mesenchyme.

NAEGELI'S THEORY

MESENCHYMAL CELL

PRO-NORMOBLAST   LYMPHOCYTE(of lymph gland)   MYELOBLAST

NORMOBLAST   LYMPHOCYTES(of the   MYELOCYTES   MONOBLAST
                                   blood)

NORMOCYTE(erythrocyte)   POLYMORPHONUCLEAR   MONOCYTE
                                              LEUCOCYTES
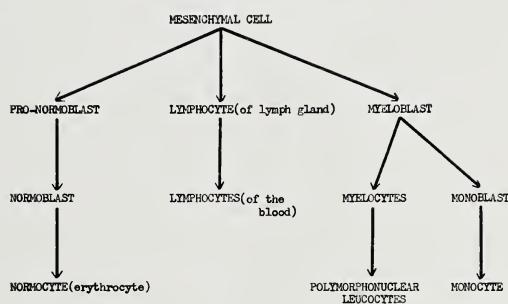
slightly modified from Sabin, Cunningham and Doan.

Figure 3. Naegeli's Theory of Blood Cell Genesis.

Figure 5. Functional chart of flight test station.

THEORY OF DOAN, CUNNINGHAM AND SABIN

MESENCHYME CELL
(embryonic)

RETICULUM   (fixed cells)   ENDOTHELIUM

MYELOBLAST   MONOBLAST   LYMPHOBLAST   (free stem cells)   ERYTHROBLAST

(various stages of maturation)

LEUCOCYTES   MONOCYTE   LYMPHOCYTES   (mature or adult cells)   ERYTHROCYTES

slightly modified from Sabin, Cunningham and Doan.
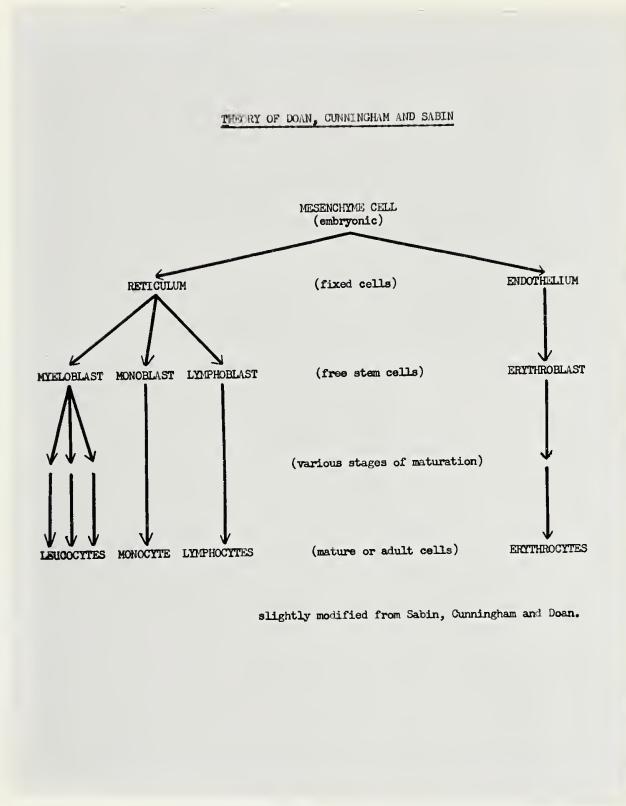
Figure 4.   Theory of Cunningham, Sabin and Doan on Blood Cell Genesis.

_2_. The Polyphyletic Theory (Dualist)

First advocated by Ehrlich, this theory maintains that the mesenchymal cells give rise to two specific stem cells which proliferate and mature, one giving rise to the white cells and one to the red.

Naegeli felt that the common undifferentiated stem cell is represented only briefly in the embryonic mesenchyme and that the derivations of these, the pronormoblasts (erythroblasts), and myeloblasts are the true stem cells, the source of the adult blood cells. He also believed that the lymphocyte was derived directly from the mesenchyme cells through a series of undifferentiated cells to the mature cell, the mediators being the cells of the lymph gland.

The theory of independent origin of red and white cells which maintains that each comes from a different type of fixed tissue cell without the intermediation of a common stem cell is based on the work of Doan, Cunningham and Sabin (19). They presented evidence indicating that proliferation intravascularly of endothelium produces erythroblasts which in turn proliferate and mature to produce erythrocytes. White cells are formed from a primitive cell which is produced by the fixed reticular cells.

The large majority of mature cells trace their existence, not through many steps of maturation from the undifferentiated reticular or blast cell but from a fairly mature cell; the

myeloid cells from the myelocytes, and the erythrocytes from the various stages of normoblasts, stages in which the ability to proliferate is retained (16). Under stress of cell depletion, all of the earlier differentiated stages, the undifferentiated blast stage or even the reticulo-endothelial stage may become active, producing large numbers of cells which begin to mature and at the same time proliferate mitotically until the normal conditions again prevail.

There is a less well known "trialistic" theory, supported by Schilling, Aschoff and Kryono (64) which maintains that there is one primitive cell for erythrocytes and lymphocytes, one for myelocytes and one for monocytes.

C. The Location of Cellular Production and the Introduction of Mature Cells to Circulation

There is little doubt, from the reports of many investigators, and no contrary evidence, that the myeloid cells of the blood are produced in the extravascular tissue of the bone marrow and by their own motility, pass through the endothelial walls of the capillaries and sinuses into the circulation. However, there is much disagreement concerning both the locus of formation of mature erythro-cytes and the mode of their passage into circulation, and the closely associated puzzle of the nature of the marrow circulation. The three

predominant views concerning the circulation were well reviewed by Doan (18) and are mentioned here briefly.

1. The earliest observations on the bone marrow by Hayer (1869) and Rudfleisch (1880) showed that the capillaries or blood sinuses are devoid of endothelial lining, simply channels or spaces in the marrow tissue, around which erythropoiesis occurred. This view was later shown to be quite incorrect.

2. Many workers observed a continuous endothelial lining, making the blood spaces not simply "holes" but complete, intact vessels. Most of the early observations were made on avian marrow, by Langer, Bizzozero, Denys and Van der Stricht to mention only a few; some of these workers, notably the last, felt that mammalian marrow differed in that the endothelial lining was incomplete, with communicating openings into the parenchyma. Drinker, Drinker and Lund in 1922, and some time after, Doan, came to the conclusion that in mammalian marrow, the endothelial lining is an intact layer of nuclei and cytoplasmic extensions. They suggest that occasionally during active hyperplasia, the thin membranous walls may become briefly interrupted due to the pressure of proliferating red cells.

3. As suggested in theory No. 2, in some cases, the endothelial lining was thought to be incomplete with spaces allowing the passage of cells in both directions between the sinuses and the parenchyma. One author even compared the marrow circulation to that of the spleen.

The functional circulation of the marrow consists of
sinusoids, thin-walled vessels which can dilate to the size of a
vein or contract to the size of an endothelial cord. The latter,
with negligible circulation, represents the active centre of erythro-
poietic activity. Most of the blood spaces are in the collapsed
state and connect with sinuses or dilated vessels, the condition
being interchangeable and facilitating the delivery of mature cells
to venous channels.

There are two views concerning the location of erythro-
poiesis in the mammal. The general concept that both leucogenic
and erythrogenic centres are extravascular was accepted by many
workers. Banting (79) felt that this was true and mature red cells
rapidly pass into the sinuses through permanent spaces in the endo-
thelial wall. Maximow theorized that red cells developed originally
from smaller endothelial cells, the development occurring just extra-
vascularly. The wall of the sinus or capillary then briefly opened
and allowed mature cells to enter.

Most workers have concluded that the erythropoietic
activity is intravascular. The very early free red cell precursors
(pro-erythroblasts) are almost absent in the normal marrow -- the
proliferating normoblasts line the inner walls of the capillaries,
or collapsed blood spaces, while the mature offspring move towards
the centre of the lumen. The pressure of increasing normoblasts
and erythrocytes causes the capillaries to dilate resulting in cell
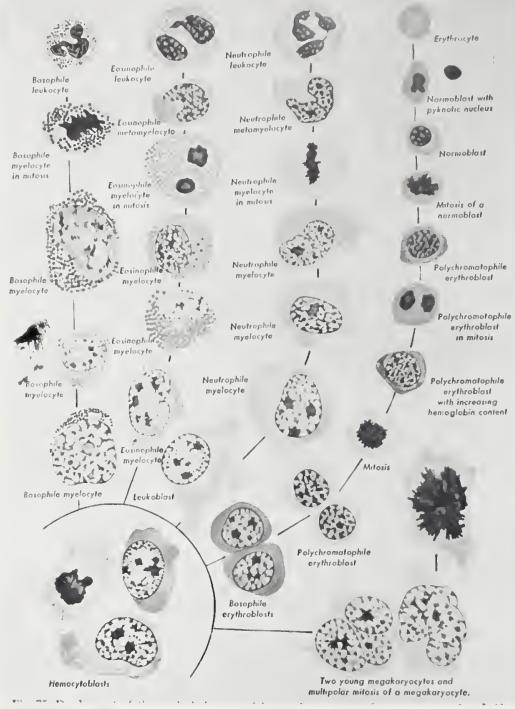
Figure 5. The Maturation of the Cells of the Blood.
From Maximow and Bloom's "Textbook of Histology."

bundles or cylinders with the mature cells in the centre and the
immature at the periphery. The mature red cell, being inert, cannot
enter circulation by its own motility as can the white cell. It
has been suggested that the growth focus or pressure of proliferation
forces the mature cells from the end of the capillaries into the
circulation. The alternative, suggested by Sabin (79), is that the
capillary dilates and a flow of plasma sweeps the mature cells into
circulation. Key's work (36) showed that immature red cells are
sticky and tend to agglutinate and adhere to foreign tissue, which
could be the mechanism that prevents immatures from entering the
circulation.

D. Maturation

A detailed description of the maturation of leucocytes
and erythrocytes is unnecessary. It is well established that the
immature myeloid cells become differentiated by the assumption of
granules, and the loss of basophilic cytoplasm and mitochondria.
The age of the mature polymorphonuclear leucocyte is indicated by
the number of lobes in the nucleus. The maturation of the erythroid
cells also follows a well established pattern, with a change in the
cytoplasm from basophilic to polychromatic and then to eosinophilic,
and reduction of the size of the nucleus.

The manner of transition from the normoblast to the
erythrocyte stage is a subject which is wrought with controversy.

There are three predominant theories:

    <u>1.</u> The nucleus becomes pycnotic and breaks down, the reticular material of young erythrocytes being the remains.

    <u>2.</u> The nucleus is expelled through the cell wall leaving the cytoplasm behind as a mature erythrocyte.

    <u>3.</u> The normoblast produces repeated cytoplasmic buds which break free as mature erythrocytes.

      The first two theories have long been considered the important ones. However, both are formulated from a combination of pure reasoning and discontinuous or interrupted observations on fixed preparations. Most writers on erythropoiesis avoid the issue by mentioning neither theory, or else, refer in a few words to one or the other theory without listing the source of information. Isaacs in Downey's "Handbook of Hematology" (20) briefly reviews the two theories. The nuclear breakdown theory was originated by Kolliker and supported by Naegeli, Neumann, Israel and Pappenheim, Schridde, and Blumenthal. The extrusion theory was originated by Rindfleisch and supported by Maximow, Jolly and others. Isaacs' review concludes "there is no definite data which indicates the exact method of denucleation."

      Cytoplasmic budding was first theorized by Malassez in 1882 and subsequently observed by many workers who attached to it
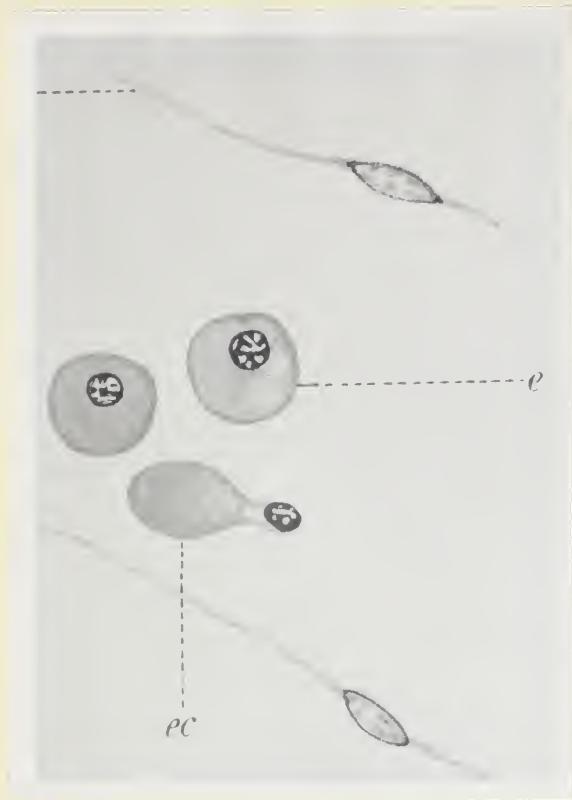
Figure 6. Erythrogenesis
          by Budding.

e - normoblast

ec - erythrocytic bud

          - from Emmel.

Figure 7. The Post-budding
          Stage.

The appearance of the normo-
blasts after the new erythro-
cytes have become detached.
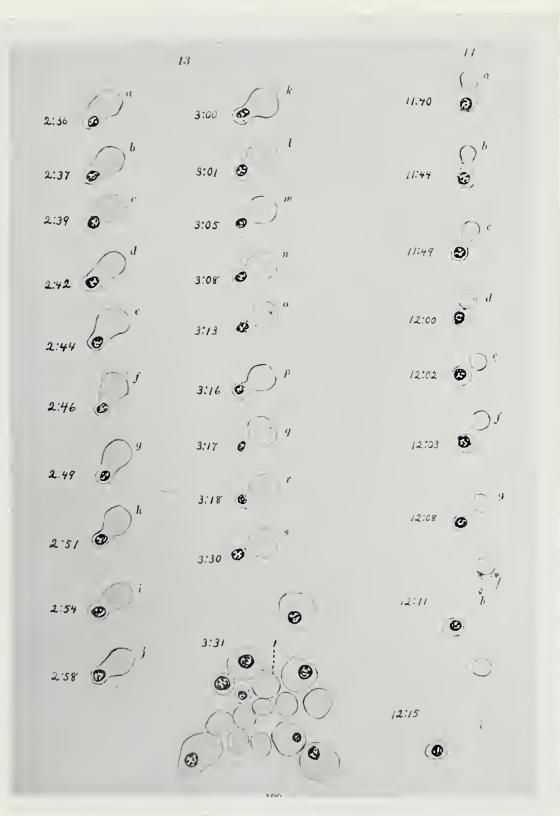
          - from Emmel.

Figure 8. Budding and Cytoplasmic Constriction.

The sequence of steps in the formation
of a mature erythrocyte from the normoblast with
times shown. — from Emmel.

various divergent conclusions. It remained for Emmel (22, 23, 24) to present results which seem to prove conclusively that mature red cells are produced by budding and cytoplasmic constriction of normoblasts. From a series of observations made directly and continuously on hanging drop cultures of blood of pig embryos and from studies of smears and fixed preparations of embryo cultures, he presented several long papers, with many drawings, describing in detail the process of erythrocyte maturation.

During the years since Emmel's work appeared, no conclusive evidence has been contributed to either the extrusion or the disintegration theory. However, in the past five years, several authors have published evidence which seems to substantiate Emmel's observations and conclusions. Bostrom (3, 4) made many observations which indicated that the manner of erythrogenesis is by budding. Plum in several papers gave evidence of normoblasts giving rise repeatedly to mature red cells (69) and actually observed the process in his micro culture chamber (68). He was able to inhibit cell mitoses with colchicine and still obtain an increase in red cell population "in-vitro." Ralph (75) using dark field microscopy photographed the process of budding. Saltman (80) also admitted its occurrence but suggested that it was an atypical occurrence characteristic of a pathological or abnormal condition.

Of the first two theories mentioned, maturation by nuclear expulsion has been in greater favour. Several writers
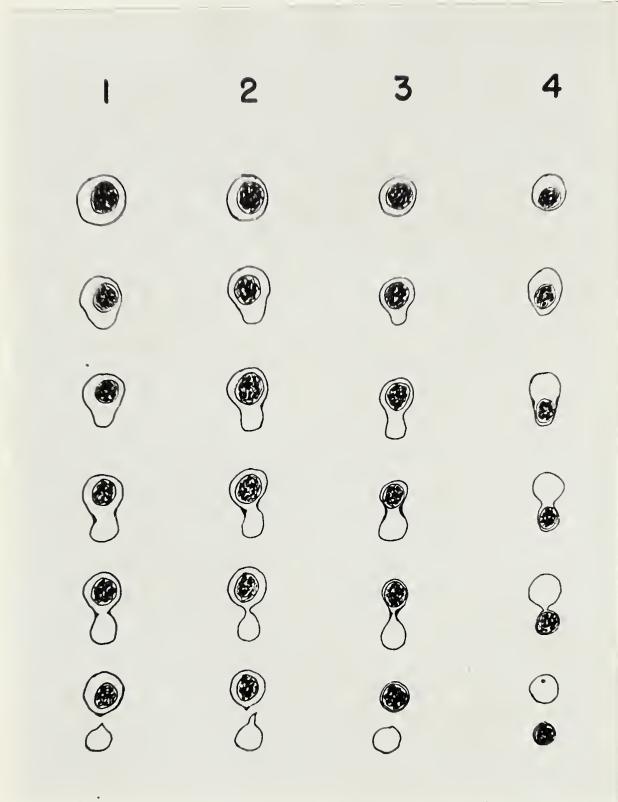
Figure 9. Compatability of the Nuclear Extrusion Theory

and the Budding Theory.

Columns 1, 2, and 3 all represent budding.
Column 4, which is an identical process, might be inter-
preted as nuclear extrusion.

including Emmel have suggested a line of reasoning which would
make that and the theory of budding compatible. The manner in which
budding occurs produces a cytoplasmic non-nucleated body and a
nucleus with cytoplasm in varying amounts surrounding it. Often,
the normoblast is small enough so that the "bud" breaks away leaving
very little or no cytoplasm with the nucleus. This results in an
erythrocyte and a nucleus surrounded by a closely adhering and almost
invisible cytoplasmic membrane. In this case, the process could be
interpreted as either budding or nuclear extrusion. It is of interest
to note that, contrary to popular beliefs, Rindfleisch, who originated
the theory of nuclear extrusion, actually observed and recorded thin
circles of cytoplasm around the supposedly extruded nucleus. His
drawings of a nucleus being extruded (drawn from a fixed preparation)
bear such close resemblance to later drawings of cytoplasmic budding
that one might conclude that what he described as extrusion was
actually an example of budding (24).

III. SURVEY OF BONE MARROW CULTURE METHODS

A. Introduction

Since bone marrow was first cultured forty-five years ago, several general methods have been employed, and modifications of these are as numerous as the investigators. In view of this, a critical survey of previous methods has been undertaken.

The general type of marrow culture and the subdivisions of each are briefly considered, followed by a review of the various classifications of media and their applications, a description of methods relating parallel steps of the techniques of various researchers; the source and preparation of the marrow, the initiation and some of the conditions of the culture.

B. General Considerations

Bone marrow can be cultured by three general methods: (1) an explant of marrow tissue can be imbedded in a plasma clot and incubated; (2) marrow tissue can be homogenized and cultured as a suspension of cells in fluid medium; (3) a suspension of cells can be introduced into a living organism and later withdrawn for study.

1. The Solid Culture

The explant or solid or clot culture method was the type utilized by researchers until, but rarely after 1936. It
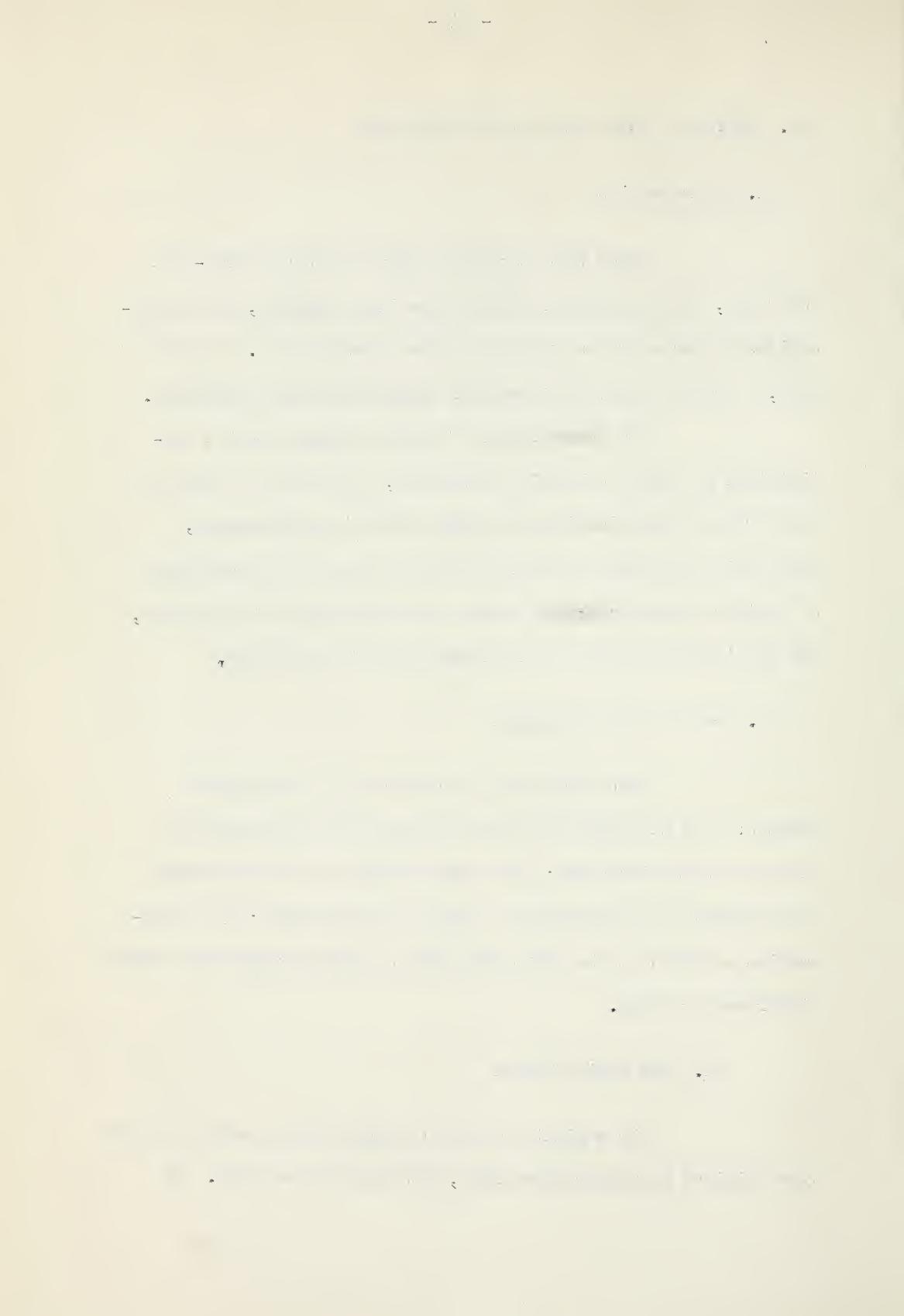
consists of placing a tiny piece of bone marrow in a mixture of plasma, physiological saline and embryo extract which then clots. After a certain period of incubation, the original explant is discarded, re-imbedded, or left undisturbed and the cells which have migrated from it, often after further incubation, are studied by histological methods.

Carrel and his co-workers incubated the explant and clot on a mica cover-slip (10), and in later work (11), in a special flask in which the clot was covered with a fluid medium which could be changed without disturbing the tissue. Maximow's method consisted of coagulating the explant in a drop of plasma on a cover-slip which was then inverted over a hollow slide, sealed and incubated. This was similar to the procedure followed by Foot (30) and Erdmann (25). Rachmilawitz and Rosin (74) simply cultured marrow fragments imbedded in small tubes of clotting plasma.

2. The Fluid Culture

Since 1936, almost all bone marrow culture studies have been carried out using a suspension of cells in fluid medium. Hematopoietic marrow tissue is homogenized with a fluid medium, the fat and fibrous matter removed, and the resultant homogenous cell suspension is incubated. Studies can be made on samples withdrawn at intervals.

This method of marrow preparation was devised by Osgood and his associates (52, 53). Their cultures were carried out

in a large complex apparatus which supplied all the functions of the
kidneys and lungs making possible continuous culture of marrow cells
in medium with accurately controllable pH level, gas composition,
osmotic pressure and removal of metabolites. (A similar apparatus
was later used with great success by Plum (67). However, because
of technical difficulties, Osgood abandoned his initial complex
culture apparatus in favour of a "simple technique" (53, 54) which
subsequently was employed by many other workers. Cell suspensions
were incubated in small vaccine vials, exact control of the various
variable conditions being sacrificed for simplicity.

To facilitate direct observation of cells in culture,
Plum (67) developed a new culture chamber which allowed prolonged
incubation of cell suspensions in an unchanging fluid medium directly
under the high power objective of a microscope. This was a similar
though more advanced version of Emmel's hanging drop for the study
of embryonic blood (22).

3. The "In-Vivo" Culture

The third general method has never been extensively
pursued. While it has no direct connection with the experimental
work of this thesis, it is of indirect interest and will be briefly
described. It involves the use of a living organism as an incubator
and culture medium. Tissue, properly prepared, is introduced into
some part of an animal and later, is removed for study. Plum (71)

injected minute amounts of marrow cell suspension into the camera
anterior oculi of rats, and after a certain period of time had elapsed,
removed and studied the contents. Pierce (63) incubated leukemic
leukocytes on the chorio-allantoic membrane of chick eggs.

C. Media

To remove tissue successfully from its native
environment to a foreign situation for the purpose of study by
"in-vitro" culturing, two very important points must be considered.
The new environment has to have a chemical composition which will
make it identical to the native environment with respect to pH,
osmotic pressure and cation and anion concentrations. It must also
be rich enough in nutritional material, so that a sufficient supply
is available to support cell metabolism for the duration of the
culture. All these conditions must be fulfilled, in spite of the
variable conditions of temperature and gas composition and accumula-
tion of metabolites, variabilities which were not present in the
original habitat.

A third, and somewhat questionable necessity for
medium is the ability to supply a framework or solid base on which
the cells can grow. It was long thought that all growing cells need
something on which to hold and to spread along. Carrel (14) and
Fell (28) are only two of those who support this idea. However, in
the last few years, the idea has been losing ground; it has been

shown that many cells can survive without any surface or framework (31). In blood cultures, cells grow well suspended in a fluid medium (54).

Media can be classified as nutritive and non-nutritive, each can be subdivided into natural and synthetic.

1. Non-Nutritive Media

The non-nutritive media find their greatest use as diluents. They are added to such nutritive media as plasma, serum, and tissue extracts to increase the available volumes, and supply buffering capacity at the desired pH.

All of the non-nutritives now in common use are synthetic.

(a) Synthetic

The development of synthetic media was begun late in the last century when many physiologists were striving toward a workable perfusion fluid for the study "in-vitro" of heart, muscle and intestinal activity. The classical work of Sidney Ringer (77) with amphibian tissue resulted in the knowledge of the mutual antagonisms of Na, K and Ca salts. He developed a solution, based on his "Balanced Salt Principle," composed of $NaCl$, KCl, $CaCl_2$ and $NaHCO_3$.

In 1901, Locke (60) modified Ringer's solution for use with mammalian tissue and added glucose to give a concentration of 0.1%. This solution is known as the Ringer-Locke solution and is often erroneously referred to as simply 'Ringer's.'

The basis for almost every saline medium now in use is Tyrode's solution. In 1910, Tyrode published a paper in which he suggested some modifications of the Ringer-Locke solution; the addition of $MgCl_2$ and NaH_2PO_4 and a pH of 7.4 to 7.8. Gey and Gey (31), in an extensive discussion, stressed the importance of a strictly uniform medium of known composition. Noting the ever present biological variation in natural and animal media, they expressed the need for a completely synthetic substance in which to culture animal material. After an extensive review of the literature, they derived a balanced salt solution with ion concentrations identical to those in serum and plasma, for use in equilibrium with a physiological pressure of CO_2.* Osgood with reference to his medium wrote in an early work "the composition of the medium is the same as that found by the Geys to be ideal for the growth of human tissues."

In most of the work reviewed, the use of either Tyrode's solution or Geys' balanced salt solution was recommended. Some utilized glucose free Tryode's solution.

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*The maintenance of pH depends largely on the buffer pair $\frac{H_2CO_3}{NaHCO_3}$. The concentration of H_2CO_3 in the solution is related to the partial pressure of carbon dioxide in the gas above. Therefore, in a solution with a fixed amount of $NaHCO_3$, any change of CO_2 from the calculated pressure will result in a change of concentration of H_2CO_3 and thence a change in the pH.

(b) Natural

It is difficult to conceive of a non-nutritive
media from a natural source. Animal tissues whether solid or fluid
in addition to the balanced salt qualities have nutrient value and
are therefore mentioned under that classification.

2. Nutritive Media

Nutritive media are used in pure form or in conjun-
ction with a non-nutritive media. Their purposes are to supply
materials which can be metabolized by the cells in culture, the
"food," and in addition, to contribute substances such as growth
promoters, vitamins and trace elements, factors which are minute
in quantity but very necessary to the processes of living tissue.

(a) Natural

The most commonly used nutritive media are the
fluid components of the blood; the plasma and serum.

Serum is the most easily obtained and the most
stable of the nutritive media. It is employed largely as the fluid
component of fluid-solid cultures, and as a suspending medium for
fluid cultures. As the result of a paper by Carrel and Ebeling (13a)
which described the inhibiting action of serum of old individuals,
Gey and Gey (31) initiated the use of human cord or placental serum
in place of the adult serum. Many workers have, for the same reason,

utilized the serum of very young animals. At least one worker (32) used reconstituted serum.

Plasma is used largely in cultures where a framework or microscopic supporting structure is necessary for cell proliferation. It is the basis of almost all solid cultures. For the same reason as was previously mentioned, foetal or placental plasma, or plasma from young animals is often used.

Rarely, other nutritive animal fluids have been used; Ascitic fluid by Loeb and lymph by Harrison (60). Tissue extracts have been employed extensively in the culture of tissues "in-vitro" but no report of their use in fluid culture of hematopoietic tissue can be found. Alexis Carrel (9) in 1913 made the first attempts at using tissue extracts and tissue juices. He observed a stimulating affect with many, but most markedly with the extract of embryonic tissue. Subsequently, extract, usually of chick embryo, but also bovine embryo has been used almost universally especially with plasma to provide a solid culture. (Many investigations dealing with the nature and locus of the stimulating substance have been reported, but as late as 1947 theories were still being presented as to the identity of the active principle (82)).

(b) Synthetic

A synthetic medium is one which contains a number of components all of which are of known composition. In other words,

the entire composition and structure of the medium must be known and accurately reproduceable.

The development of such a medium was begun early in this century and since then, numerous workers have entered the field. In the last ten years, efforts have been increasing, with the work of Fischer et al (29), and Morgan, Norton and Parker (42) most prominent. Fischer and his group devised a mixture which contained all the substances known to be of biological importance in animal tissue. Their mixture U-605 contained forty-nine elements including salts, sugars, amino acids and vitamins. By removing one component at a time and studying the results, they were able to reduce the number of elements to twenty and still maintain good growth. Morgan, Norton and Parker established roller-tube cultures and then substituted artificial mixtures for the standard nutritive medium. After a great deal of work, they arrived at a totally synthetic mixture containing over sixty components.

These mixtures, although inferior to natural nutritive media, are the basis for further work which will result in the availability of a nutritive media of unchanging and known composition.

3. Special

Several substances, being closely associated with the use of tissue culture media should be discussed in this section.

Anticoagulents form an important part of tissue culturing of blood and bone marrow. The chemical anticoagulents, citrate salts, were in use occasionally in the preparation of plasma, and in the preparation of the culture. Osgood used a 0.5% citrated balanced salt solution with which he made the initial marrow cell suspension. Plum (68) did the same using 3% Na citrate solution.

There are indications (72) that citrate solutions are toxic to nucleated blood cells "in-vitro," and that heparin is preferrable. Heparin has been widely used in tissue culturing. It has the advantage of being active in extremely small concentrations. Gunz (32) in the culture of leukemic blood used a 1:15,000 concentration. Israels (34) used heparin 1:6,000 in the initial suspension and centrifuging of marrow. Pierce (62) used 1:25,000 in the culture of leukemic blood. Feinmann et al (26) avoided heparin because of some evidence that it inhibited mitoses.

To provide support for cell growth "in-vitro," agar and gelatin have been tried, and in the absence of plasma, a suspension of fibrinogen (10). These were never as successful as the plasma clot. Digestion of the clot by trypsin frustrated early attempts to maintain cultures of some tissues for extended periods. The addition of a little egg yolk or sodium linoleate (12), both demonstrating antitrypsinic activity, prevented this.

To discourage the growth of bacteria, penicillin has been added to culture media. Gunz (32) used 100 units per ml. of culture medium.
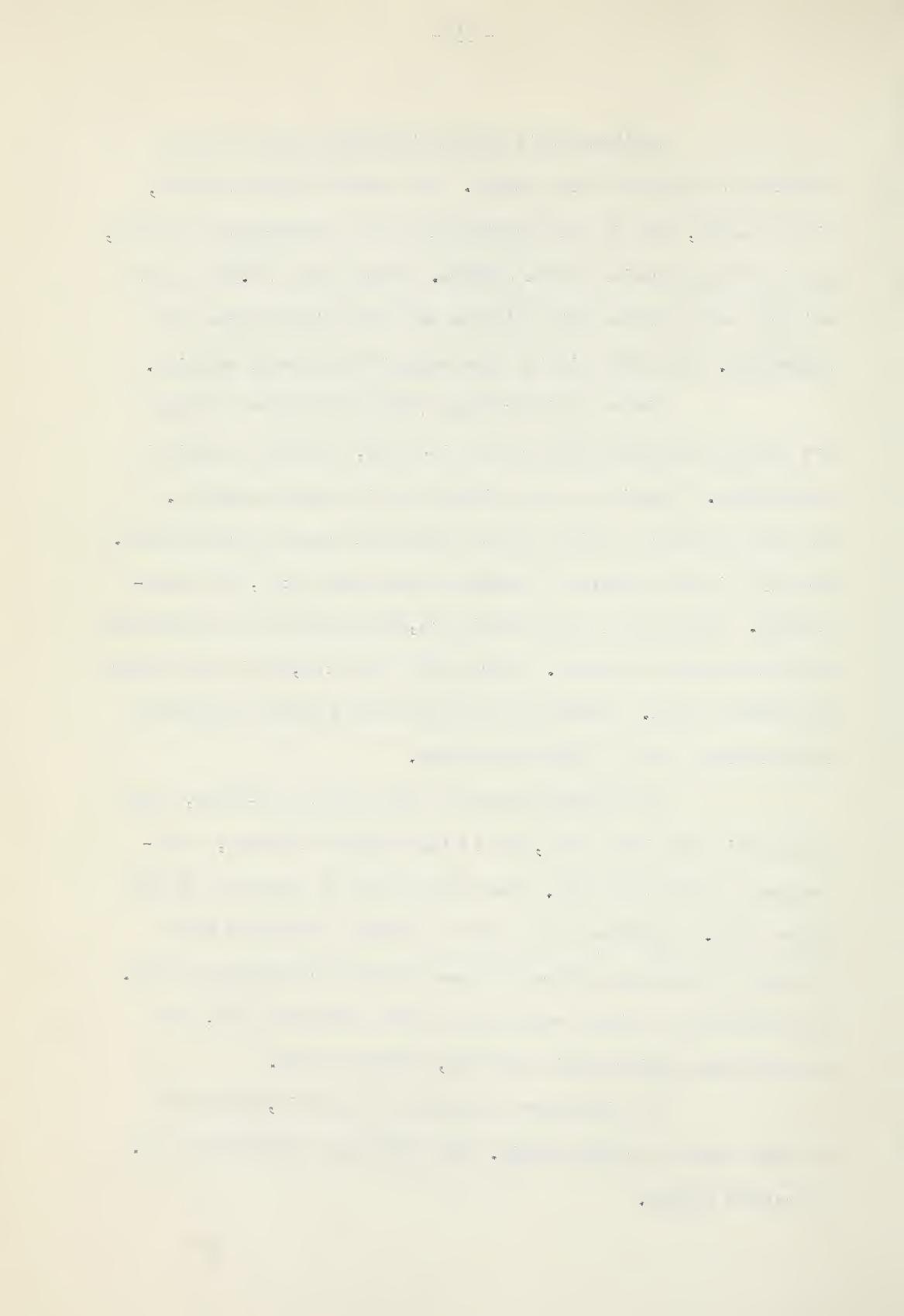
D. The Preparation of the Culture

    1. The Explant or Solid Culture

        Until 1936 all investigators utilized small animals
as the source of hematopoietic tissue for culture. Carrel and
Burrows (8a) using explants of cat femoral marrow and later Foot (30)
and Erdmann (25) using chicken femoral marrow teased out bits of the
tissue into Locke's solution. The cultures were prepared by placing
a bit of the bone marrow into a little plasma on a cover-slip which
in turn was inverted over a hollow slide and sealed producing a
hanging drop preparation which was incubated at a temperature of 38
(Erdmann) or 40 (Foot) degrees centigrade.

        Some workers, studying more mature cells, used
peripheral blood. Carrel and Ebeling (10) centrifuged chickens'
blood in paraffin tubes at a high speed for 10 minutes, pipetted off
the plasma, and placed several drops of embryo extract on the buffy
layer of cells. Fifteen minutes later, this layer, now coagulated,
containing the nucleated cells of the blood, could be lifted into a
small container of Ringer's solution, washed free of erythrocytes,
and cut into small pieces which were subsequently cultured as follows.
A bit of this white cell coagulum was placed on a cover-slip in a
drop of medium consisting of plasma, or plasma and Tyrode's solution,
and embryo extract, or twenty percent fibrinogen solution instead of
plasma. The medium soon coagulated producing a solid clot culture,

which was incubated. Every few days, the coagulum was cut into pieces
with a sharp scissor and each piece placed into a new medium as before.
Pierce (62) followed a very similar procedure. The bits of leucocyte
film were placed in a medium of rabbit plasma, Tyrode's solution and
embryo extract which clotted; the cover-slip to which the clot was
attached was then inverted over a hollow Maximow slide.

Carrel and Ebeling, in a later work (11), used a
dual phase solid-fluid culture to study the action of serum on lympho-
cytes. On the bottom of a special flask about five centimeters in
diameter was placed a fragment of spleen or white blood cell coagulum
with a very thin layer of clotting medium in which the cells would
proliferate and mature. As a source of nutrition, a small amount of
fluid medium, enough to thinly cover the clot, was then introduced and
the flask placed in an incubator. There was a continuous diffusion
of gases, nutrients, and metabolites between the fluid phase and the
solid phase of the culture. Every three or four days, depending on
its condition, the fluid phase was replaced without disturbing the
solid phase or the cells within it. By this method, the cells could
be kept for long periods in culture without the usual involved procedure
of subculturing.

Rachmilawitz and Rosin (74) simply embedded explants
of tibial marrow of young rabbits in tubes containing clotting medium
and incubated at 37° C.

2. Fluid Cultures

Coincidental with the introduction of fluid suspension cultures for blood forming tissue, Osgood and his associates (54) began to use human sternal marrow, this was the first recorded instance where human marrow was utilized for "in-vitro" cultural studies of hematopoiesis. From one to ten millilitres of marrow was aspirated into a syringe and introduced into twenty-five millilitres of sterile citrated balanced salt solution in a fifty millilitre tube. This was centrifuged for 15 minutes at 1,500 r.p.m. The original amount of balanced salt solution was removed and the cells were resuspended in the remaining fluid. The suspension was then introduced into a volume index tube, which was capped and centrifuged at the same speed as before for thirty minutes. At the end of this period, the supernatant fluid was discarded, the buffy "myeloid-erythroid" layer was removed and placed in eight millilitres of balanced salt solution in a thirty millilitre vaccine vial where, after a nucleated cell count, the suspension was diluted with balanced salt solution and cord serum to give a final concentration of one to two thousand nucleated cells per cubic millilitre of medium containing thirty-five percent cord serum. This suspension was cultured either in his complex apparatus or by the less complicated vaccine vial method. The latter consisted of placing about 12 millilitres of the suspension in each of many vaccine vials which were then sampled for counts and smears, stoppered and incubated at 37.5° C. without agitation. The air above the cultures was changed through the rubber stoppers with a syringe and needle.

Israels (34) was the first of many to repeat Osgood's simple technique. His method was similar to the original with a few minor exceptions. He used heparin and Parker's solution instead of citrated balanced salt solution. The centrifuging during the separation of the myeloid and erythroid cells from the other marrow components was somewhat more extreme and prolonged. The gas mixture introduced into the culture bottles was fifty percent oxygen, and three and a half percent carbon dioxide. The temperature of incubation was 38°C.

Plum (68) cultured marrow from dogs, cats, rabbits and humans. In every case but the latter, he entered the head of the femur by surgical methods and aspirated marrow into a syringe containing citrate solution. Human marrow was taken by sternal puncture. After one centrifuging at fifteen hundred r.p.m., the myeloid-erythroid cell layer was transferred to culture in his apparatus for large samples, similar to Osgood's complex machine, or into his microculture chamber for direct observation.

Norris and Majnarich (44) suspended marrow cells of various animals in glucose free Tyrode's solution and incubated in small flasks at 37° C., agitating throughout the culture period in a modified Warburg apparatus. Hays (33) utilized a method very similar to that of Osgood, though somewhat more traumatic, suspending the marrow cells from rats, like Norris, in glucose free Tyrode's solution, and warming to 38° C.

Among the many others who cultured marrow, Lajtha
(38) used a medium of thirty-five percent human serum and sixty-five
percent Gey's or Ringer's solution. Feinmann, Sharp and Wilkinson (26)
during a study of the serum maturation inhibitors used sternal marrow
of pernicious anemia patients in various ratios of Gey's solution, and
normal and pathological human serum. Gunz (32) used Osgood's method
in the culture of leukemic blood in equal parts of Ringer's solution
and reconstituted human plasma.

E. Concentration of Cells, Depth of Suspension and Duration
    of Culture

The continued existence of cells in culture depends
largely on the supply of nutritional material, the removal of meta-
bolites, and the maintenance of proper gas partial pressures, pH and
osmotic pressure. The length of the survival of the culture can
generally be said to be inversely related to the speed at which
nutritional sources are exhausted and metabolites accumulate, which
in turn, are related to the concentration of cells in suspension.
The diffusion of gases into a culture medium, whether solid or
liquid, depends upon the depth or the thickness of the medium. It is
of prime importance therefore to review, in the reports of past workers,
the concentration of cells in the suspending medium or the ratio of
medium to cellular material, and the thickness or depth of the culture
medium through which the gases must diffuse.
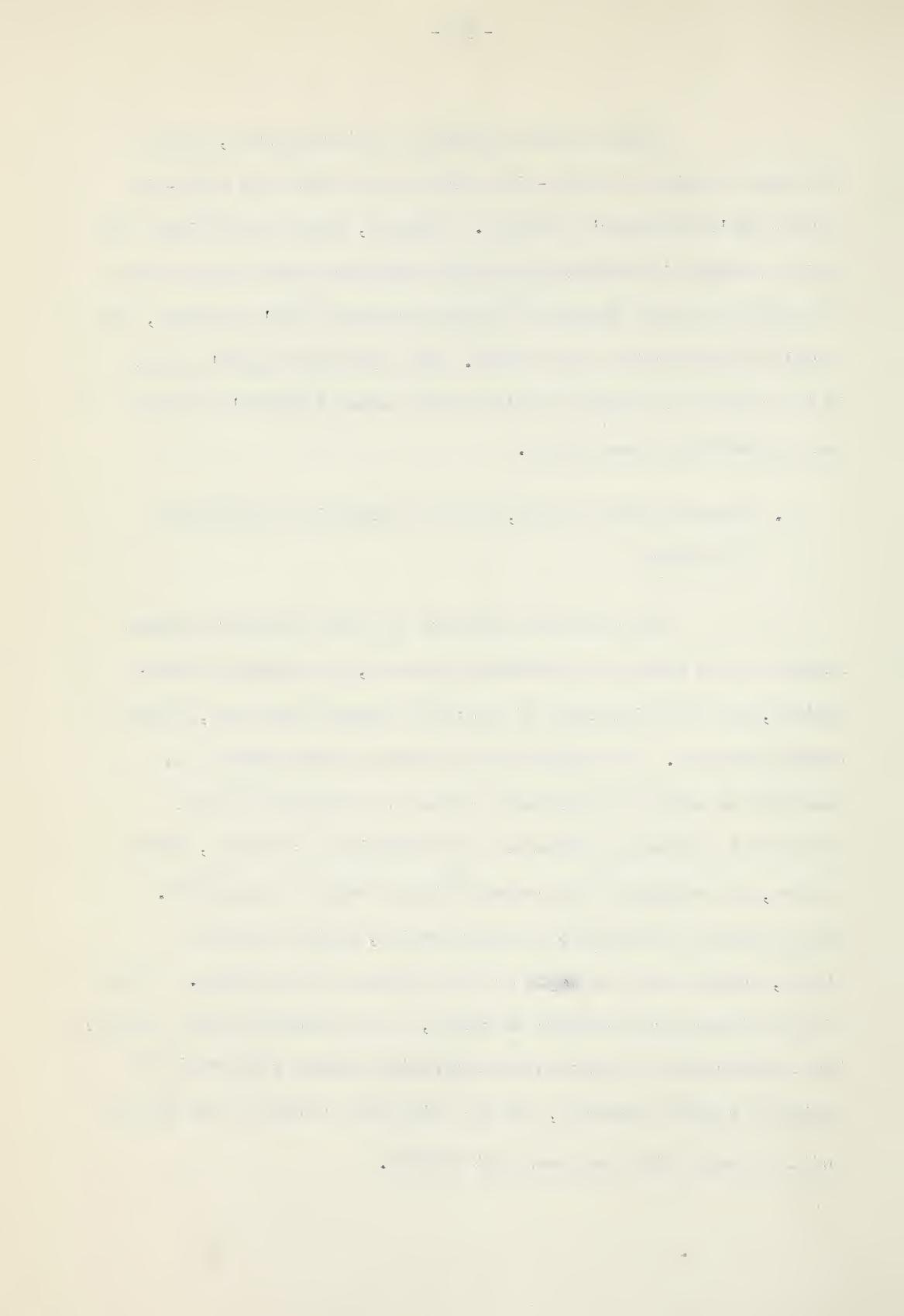
Foot's cultures (30), being hanging drop in character, were only a few millimeters thick and had a large surface area for gas exchange. There was no particular effort made at controlling the gaseous composition surrounding the clot but its volume was many times that of the culture and therefore, of almost constant composition. He cultured up to six days and reported no signs of degeneration (many changes did occur but he classified all of these as differentiation).

Carrel and Ebeling (10) prepared clotted cultures similar to Foot's. By transferring bits of the coagulum to new medium every fourth or fifth day, they were able to maintain their first pure strain of mononuclear leucocytes for almost three months. There is no mention of attempts to control the composition of gases surrounding the culture. In later work (11) the same authors, using the solid-fluid culture system, were able to maintain motile blood cells for over a month, and other cells for much longer periods by frequently changing the fluid portion of the medium. The thickness of the coagulum, the solid portion of the culture, was not much more than one millimeter and covering fluid component was about the same depth.

Rachmilawitz and Rosin (74) embedded tissue in tubes of clotted medium fifteen millimeters long by eight millimeters in diameter, providing a very small area for gas exchange. Their paper records the presence of, but not the degree of degeneration after cultures of up to five days duration.

Osgood and Brownlee (54) found that the ideal concentration of nucleated cells in fluid suspension is around two thousand per cubic millimeter, with the medium being changed every forty-eight hours. Any increase in cell concentration requires a parallel increase in the frequency of the medium change (58). As a result of their earlywork, they were able to suggest that cultures survived at pH's from 7.0 to 8.0, the optimum being 7.4 to 7.8, and osmotic pressures equal to sodium chloride solutions of 0.7 to 1.0 gms. percent with slightly hypotonic medium the best. Temperatures from just above freezing to forty-one degrees centigrade were tolerated with the rate of multiplication and maturation directly related. Cultures were maintained for two weeks at room temperature without changing the medium.

Plum (68) recorded cell concentrations of thirty to fifty thousand per cubic millilitre with the nucleates varying from one to ten thousand. Although the duration of his cultures was limited to six hours, the continuous replenishment of the culture medium and removal of metabolites through a system of semipermeable membranes of his equipment suggests that prolonged cultures could easily be a reality. Gas tensions were kept constant by a steady flow of filtered carbon-dioxide-free air which bubbled directly into the cell suspension. The rate of flow was adjusted to maintain the proper oxygen supply and allow the optimum amount of carbon dioxide to accumulate in the culture medium.

Feinmann, Sharp and Wilkinson (26) cultured a suspension of cells of fifteen to twenty thousand nucleates per cubic millimeter in a layer seven millimeters thick for forty-eight hours.

Gunz (32) in culturing leukemic blood incubated for six days without changing the medium. The fluid layer was never more than one centimeter thick and the concentration of cells was around two thousand per cubic millimeter.

F. Methods of Study

The investigator who cultures cells "in-vitro" is interested in one or more of three possibilities; (1) the maintenance of a pure strain of cells, unchanging for some time; (2) the changes or absence of changes in the appearance (morphology) of cells in culture; (3) the increase or decrease in the total cell population or any component of it.

1. The Maintenance of Unchanging Strains

The maintenance of a multiplying, unchanged strain of cells has been accomplished by Carrel's flask method although with less success with blood cells than other tissues. It allows a series of experimental tests to be done without the contention of biological variation in the cultured tissue. No reports have been published concerning the long term maintenance of pure strains in fluid medium.

2. Morphological Studies

To investigate the morphological changes in blood cells, the investigator must make studies before and after incubation.

The solid culture by its very nature must be terminated before detailed study can be initiated. The usual procedure consists of preparing a large series of identical cultures, and fixing several after various periods of incubation. Detailed examination must be preceded by fixing and staining "in toto" or sectioning and staining. Both of these methods, especially the latter, tend to introduce artifacts which endanger accurate observation.

Fluid cultures have certain advantages in morphological investigations. The culture can be studied frequently and its progress carefully followed without disturbance, by a process of sampling. Many hematologists (31, 58) prefer smears of fluid suspensions to fixed preparations since the nuclear characteristics, the basis of cell differentiation, are more easily seen. Moreover, studies of solid cultures are often difficult due to the overgrowth of fibroblasts, a phenomenon which seldom occurs in fluid cultures.

There are certain disadvantages in the fluid culture technique. Structural relationships are entirely lost; the process of sampling and concentrating cell suspensions before smearing often encourages the formation of artifacts.

Morphological studies by direct observation
requires some mention. Hanging drop preparations and microcultures
are of decided value in investigations of mitosis, normoblast
maturation and similar problems where continuous observation is a
requisite. However, the fact that the cells must be examined unstained
except where vital stains can be used makes accurate observation diffi-
cult.

3. Population Changes

The appreciation of proliferation of cells cultured
in a solid medium is not possible by a direct method. Parker (60)
reviewed the available procedures which include: measuring the area
of the culture; estimating mitotic coefficients; measuring dry weights;
metabolic measurements by monometric methods; measurement of glucose
utilization or lactic acid production or various other metabolic
determinations; the utilization of radio-active isotopes. The dis-
advantages of such indirect methods are obvious.

The concentration of cells cultured in fluid media
can be estimated with reasonable accuracy by the use of the hemo-
cytometer. Repeated counts at various intervals can be done without
disturbing the culture as a whole. In conjunction with differential
counts on stained smears, the concentration of any type of cells in
a culture at any time can be ascertained.

G. Summary

Live tissue has been cultured in the past by three general methods, the solid or clot culture, the fluid culture and the "in-vivo" culture. In the study of blood and blood forming tissue the fluid culture has, since its inception, been utilized far more extensively than the other two methods.

Tissue must be removed to an environment which is identical with the native environment and which is rich in nutritional materials. The former need is fulfilled by non-nutritive media, generally mixed salt solutions based on Tyrode's classical formula. Nutrition can be supplied by such natural substances as serum, plasma, and embryo extract (use of the latter in blood cultures is not widespread) and several complex synthetic mixtures. Heparin is the most popular anticoagulent. Penicillin has been employed to discourage bacterial proliferation.

Marrow has been obtained from almost all small animals, chicken and rabbit tissue being most popular. In the last fifteen years many workers have cultured human marrow. With only rare exceptions all reports indicate that incubation temperatures of between 37°C. and 38°C. are most successful.

In solid cultures, explants of marrow tissue or bits of the buffy coat of peripheral blood were embedded in clotting medium. Subculturing every few days is necessary to maintain the living cells. Double phase cultures in which a covering fluid medium was changed without disturbing the clot containing the cells removed the necessity of frequent subculturing.

In fluid cultures, suspensions of cells in various
fluid media were incubated. The marrow was usually treated to eliminate
fat and fibrous material and to concentrate the myeloid-erythroid
component of the total population by repeated centrifuging and
resuspension. Attempts were made to support growth in complex
arrangements in which, by systems of semi-permeable membranes, nut-
ritional substances and oxygen were supplied and the metabolites
removed. Almost all who used fluid cultures adopted Osgood's method
of using small vaccine vials or similar containers. Few workers
attempted to control the composition of gas above the culture medium.
In fluid cultures, there are contradictory figures concerning the
greatest concentration of cells which can be successfully cultured
and the duration of culture. The thickness of the fluid medium
never exceeded one centimeter. Agitation of cultures was affected
in two cases by bubbles of gas and in one case by mechanical shaking.
Most workers report no agitation.

The culturing of tissue "in-vitro" makes possible the
maintenance of unchanging strains of cells, morphological and numerical
studies. The fluid method lends itself more to the latter and in some
cases to the other two. The solid method is favoured in long term
studies and in some morphological studies .

APPARATUS AND METHODS

I. INTRODUCTION

It is evident from the reports of previous workers
who have cultured bone marrow in fluid media, and was emphasized by
McLuhan and Nelson early in this project that the existing method
of preparation of the marrow is too long and exposes the cells to
undesirable trauma. It soon became obvious that the immediate
problem was the simplification and improvement of the method of
culturing marrow so as to provide a reliable medium for more
advanced work.

The technique was to be developed so that it would
have the following qualifications.

1. Simple and Quick. Previous processes required several hours to obtain and prepare marrow for culture. So much glassware was necessary that the task of preparing for subsequent cultures became monumental.

2. Atraumatic. Mechanical processing, notably traumatic homogenizing of marrow suspensions, and prolonged and high speed centrifuging had to be eliminated to minimize cell damage.

3. Physiological. In previous works cells were exposed to various synthetic media with the possibility of their being unphysiological and containing chemical contaminants. The long period of preparation allowed temperature changes and bacterial invasion to occur.

4. Complete. For best results, a suspension of nucleated marrow cells must be obtained, with a relatively low population of mature erythrocytes, and free of fat and debris.

This portion of the thesis describes all the technical procedures used in the experimental work, including the development of a method of preparing marrow utilizing untiluted native serum as a suspending and culture medium. The method is simple and quick requiring only a few pieces of modified laboratory glassware. The marrow can be obtained and prepared for culture in less than twenty minutes without the application of traumatic processing, there being only one brief slow centrifuging period and only slight mechanical

agitation. It is physiological since the cells never leave their
accustomed environment (serum) and the danger of accidental chemical
contamination is reduced. The procedure is dependable and complete,
yielding a cell suspension with little extraneous material even when
prepared from very fatty marrow.

II. EQUIPMENT AND TECHNIQUE

A. Accommodation

Two rooms were used throughout the project. One room with dimensions of 15' x 15' was arbitrarily subdivided, half being used as a cleaning-sterilizing area and the other half as the operating-culture-counting area. For the latter, a very small dust-proof room with sealed windows and a sliding door would have been preferable, but was not available. The second room was used for animal preparation, non-sterile animal operations and for histological (technical) work. In addition facilities were available for photography, glassblowing and wood and metal machining.

B. The Use of Siliconed Surfaces

The glass which was to come in contact with cells, the culture flasks and marrow preparation tubes, were treated with a silicone preparation (General Electric - "Dri-film," 9987) to produce a smooth, low adhesive index, non-wetting surface. Cairns and Lajtha (7) indicated that this prevented the loss of nucleated cells by sticking, and Feinmann et al (26) stated that it was of advantage in maintaining the nucleated cell population.

The silicone was applied to scrupulously clean glass by electrically driven rotary buffer with which all parts of the surface were strenuously rubbed. Then the surface was rubbed with
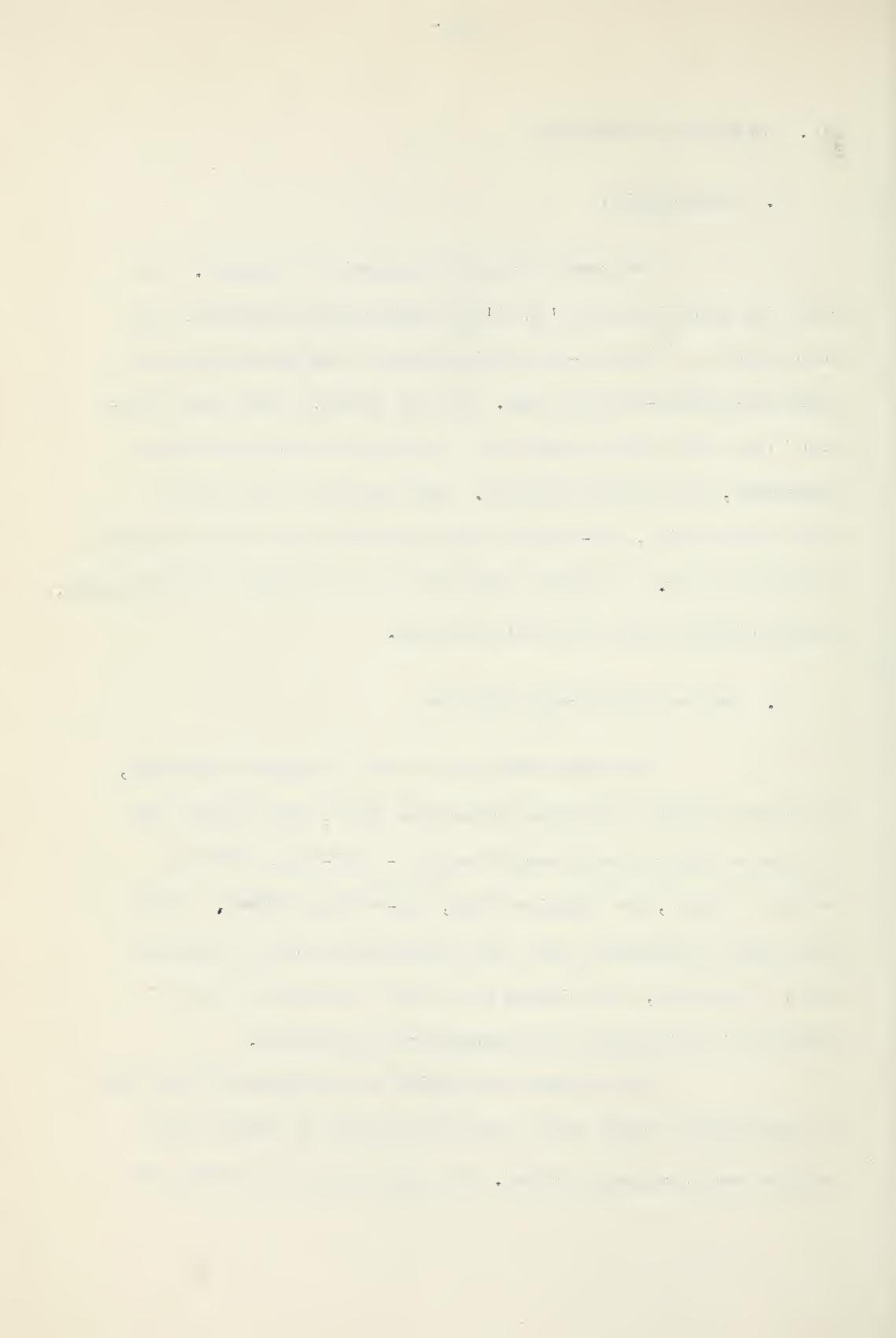
dry gauze after which the glassware was immersed in distilled water. Several hours later, the glass was drained and again rubbed with dry gauze. This provided a smooth, continuous non-wetting surface. To remove any free acids and surplus silicone the equipment was allowed to soak in several changes of distilled water for several days. This surfacing remained effective up to two months at which time the process was repeated.

C. Cleaning and Sterilizing

Because of the chemical content and oily nature of tap-water in this locality, all water to be used was distilled in a Barnstead type EL-1 metal still.

For some time, "calgonite" brand laboratory detergent was used for all glassware. However, there was some indication that traces of it, sticking to the glassware were interfering with cell survival, and causing hemolysis. In addition, its alkaline nature caused the early breakdown of silicone surfaces. Solutions of soft soap (Ivory brand) proved to be equally impractical. Therefore, a system was adopted whereby the equipment was divided into two groups. The siliconed glassware was cleaned with distilled water and towards the end of the project, with double distilled water, and extreme mechanical scrubbing with low and high speed electrically driven brushes and extended soaking in double distilled water. For unsiliconed equipment a 3 or 4% solution of trisodium phosphate in distilled

water provided good detergent activity. Since that salt is very
soluble, several rinses removed every trace. Needles and pipettes
were washed and rinsed by drawing the phosphate solution and distilled
water through under vacuum provided by a water vacuum pump. Syringes
and blood diluting pipettes were dried with acetone.

Sterilizing of all equipment was affected in a steam
autoclave (Prometheus Electric Corporation, New York) at eighteen to
twenty-five pounds pressure for twenty minutes to half an hour. Needles,
small syringes, blood diluting pipettes, stirring rods, transfer pipettes
and various other small pieces were autoclaved and stored in test tubes
plugged with absorbent cotton. Larger pieces such as syringes, large
pipettes and marrow aspirators were sterilized in bags made of several
thicknesses of linen or unbleached cotton. A few very large pieces
such as one hundred millilitre syringes were wrapped in heavy brown
paper and autoclaved. Instruments were laid out on a tray and steril-
ized in dry heat, 160° C. for one hour.

III. CULTURE MEDIUM

Early in the project, cells were suspended and processed in Gey's balanced salt solution and cultured in balanced salt solution and native serum in the ratio of two to one. The chemicals used in the preparation of balanced salt solution were the purest available.

Each salt was made up into a one litre stock solution which was sterilized through a 0.5 micron Seitz filter disc under vacuum. As the need arose and not less frequently than every two weeks the balanced salt solution was prepared by mixing exact amounts of the various stock solutions, adding a weighed amount of dextrose, diluting to one litre and sterilizing by Seitz-Filtering as before. Three drops of phenol red solution were added before filtering producing a deep red color, indicating a pH of 7.6. The bottle was stored in a refrigerator at about 4°C. and the solution was withdrawn from the bottle as needed with a sterile one hundred millilitre pipette.

Table 1. The Composition of Gey's Balanced Salt Solution. (Grams per Litre of Medium.)

NaCl	− 8.00	KH_2PO_4	− 0.03
KCl	− 0.37	$MgSO_4 \cdot 7H_2O$	− 0.07
$CaCl_2$ (anhyd.)	− 0.17	$NaHCO_3$	− 2.27
$MgCl_2 \cdot 6H_2O$	− 0.21	Glucose	− 1.00
$Na_2HPO_4 \cdot 2H_2O$	− 0.15		
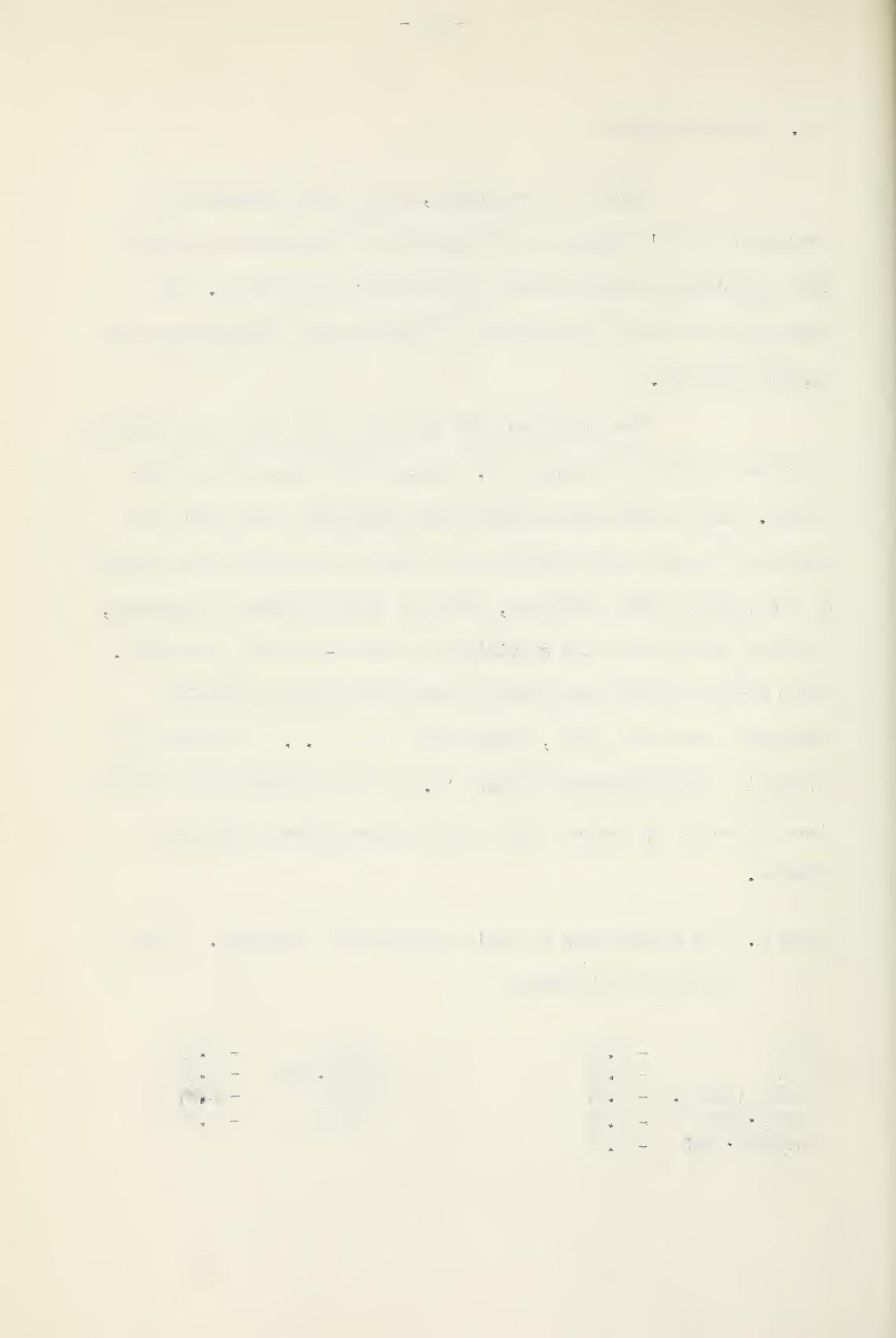
Serum was obtained from the same animal as was the marrow for culture. Blood withdrawn from the carotid artery or by cardiac puncture was centrifuged at 3,000 r.p.m. for five minutes in two 50 ml. narrow necked centrifuge tubes. The serum was removed, pooled and centrifuged again for five minutes to remove any cellular or fibrous material.

To obtain plasma, blood was withdrawn as before into enough heparin solution (Eli Lilly & Company and later Connaught Medical Research Laboratories) to provide two international units of the anticoagulent for every millilitre of blood. Centrifuging for 10 minutes at 3,000 r.p.m. resulted in the separation of the cells and plasma, the latter being pipetted into a fresh tube and briefly recentrifuged. Normal human serum and plasma was obtained from medical and dental students by puncture of the cubital vein. Pathological human serum was obtained from patients at the University Hospital. Since previous workers have found that rabbit marrow cells agglutinate and hemolyze in human serum (43), all human serum used in cultures was previously heated to 56.6° C. for a period of one hour and fifteen minutes. Although agglutination still occurred, it seemed less severe, and there was no obvious hemolysis. To fix the agglutinins, the serum was incubated at 37° C. while repeated aliquotes of washed rabbit red blood cells were added until no further agglutination occurred.

IV. MARROW SOURCE

Healthy male rabbits were used; any animal showing signs of infection, wasting condition, or an appearance of lethargy was discarded. They were kept together in a large cage and fed standard rabbit pellets and water ad lib. The average weight of the rabbits used was about 2.8 kg. with the range of weights between 2.0 kg. and 3.7 kg.

The tissue used in culture was femoral marrow. However, because of its fatty and somewhat hypoplastic nature in the healthy adult rabbit, certain problems were encountered during its preparation for culture. It was felt that it would be advantageous to treat the rabbit in such a manner as to affect a replacement of the fatty marrow to some degree by erythropoietic cellular tissue.

The methods by which an animal can be stimulated to erythropoietic activity were reviewed briefly and it was decided that chemical and administrative methods such as injections of cobalt salts, colloidal preparations, carbon suspensions and substances which cause hemolysis should be avoided for fear of interfering with the subsequent growth of the marrow "in-vitro." Three methods were considered. By simulating the anoxic conditions of high altitudes in a special box, an erythropoietic response can be obtained resulting in the replacement of fat by cellular tissue. This method was eliminated because of the technical difficulties involved. The method used by Sabin and her

associates was also considered. This consists of starving the animal until it is in an acute state of inanition, and then returning it to a normal diet. The bone marrow responds quickly showing replacement of fat with a cellular, largely erythroid element. Because this method is time consuming and often only partially successful, it was not used. The third method, the one finally employed, consists of hemorrhaging the rabbits acutely and at the height of their erythropoietic response, culturing the marrow. After considering other methods of hemorrhaging such as carotid or femoral artery cut-down and venous incision, it was decided to use cardiac puncture which had the advantages of being simple and quick, and requiring only one piece of equipment and no anaesthetic, and inflicting little mechanical damage to the rabbit.

The rabbit was weighed and the volume of blood calculated from data presented by Went and Drinker (83) who did total blood volumes on a series of rabbits and found the average volume of blood to be 8.7% by weight or 9.2 ml. of blood per hundred grams of body weight. The rabbit was then placed on the operating table and the back legs fastened down with a rope. An assistant held the rabbit so that it was stretched out, lying on its left side with its sternum parallel to, and slightly overhanging the edge of the table. When the rabbit had relaxed, the position of the heart was approximated by palpating the apex beat.

Figure 10. The Position for Cardiac Puncture.

Figure 54. The Hoofddam for Outdoor Pictures

A thirty or fifty millilitre syringe of the "Luer-Lok" type and an eighteen gauge needle were used, neither being sterile although both were quite clean. The needle was introduced into the thoracic cavity, passing between the ribs about two centimeters from the sternal margin. If the needle was in apposition to the heart, a strong thrust was transmitted to the hand of the operator with each beat. If the needle had passed slightly to one side or other of the heart, this could be sensed from the character of the thrust and the needle was partially withdrawn and the approach corrected. If no impulse was felt, the needle was completely withdrawn and the process repeated. When the needle was in the proper position, it was advanced slowly until the tip entered the heart cavity, whereupon, by the inter-cardiac pressure, blood "shot" into the nozzle of the syringe. With special care that the position of the needle remained unchanged, the piston of the syringe was withdrawn until the predetermined amount of blood had been removed. The needle was then removed by a smooth, quick movement, and the rabbit released. The blood was discarded except where special mention is made.

The rabbits, after their initial protests at being restricted and somewhat inverted by the assistant, seemed to relax completely. Aside from the prick of the needle passing through the skin which would cause them to gasp momentarily, they showed no signs of pain or discomfort. After withdrawal of the needle and release by the assistant the rabbits immediately returned to their

normal position. In some cases, after removal of as large amounts as one-fifth of the circulating volume, their movements were, understandably, a little slow, and they appeared somewhat wobbly and heavy of eyelid.

For a short while, rabbits were bled three times within a week, the total amount removed being approximately equal to one-third of the circulating volume. In a subsequent series of eleven rabbits the hemorrhaging was reduced to twice within three or four days, the total amount removed being about one-quarter of the circulating volume. For the remaining cultures, the rabbit was bled once, about one-fifth of the circulating volume being removed and the marrow cultured about five days later. In the very early instances, each rabbit was given 1μ of liver extract intramuscularly after each puncture, but this was discontinued.

Only two rabbits died as a result of the bleeding, one within ten minutes and one twelve hours after the operation. On post mortem, both showed pericardial cavities filled with large red clots.

V. OPERATIVE PROCEDURE

       The operative procedure can be discussed under three subheadings: (a) the anaesthetic and preparation, (b) obtaining the blood, (c) obtaining the marrow.

A. The Anaesthetic

       For fifteen cultures early in the project, sedation consisted of intravenous nembutal, 30 mgm. per kilo. of rabbit weight injected into the marginal vein of the ear followed by "open drop" ether. However, this method had several disadvantages. The nembutal often did not provide deep enough initial sadation, and other times, stopped respiration completely. The ether was difficult to administer in such a manner as to depress the rabbit below the pain threshhold without causing the cessation of respiration. What was more disturbing was a strong smell of ether in the serum which was to be used as culture medium.

       A series of ten anaesthetics, 10 mgm. of morphine subcutaneously or 25 mgm. meperidine hydrochloride (demerol) intra-muscularly was administered half an hour pre-operatively followed by a mixture of 25 mgm. sodium pentothal and 100 mgm. luminal per kg. of rabbit weight given in two parts 20 minutes apart. Generally, a small amount of ether was used to control the depth of sedation.

       For the remaining twenty rabbits, 25 mgm. of demerol was given twenty minutes pre-operatively. Then about 150 mgm. of

nembutal was dissolved in 1 ml. of sterile saline, and the solution
injected very slowly intravenously until the rabbit lost conscious-
ness. This was generally sufficient to keep the animal below the
pain threshhold for the duration of the operation. The dose varied
with the rabbit but usually amounted to about 40 mgm. per kg.

B. Obtaining the Blood

The anaesthetized rabbit was tied to the operating
table by its four limbs so that it was lying directly on its back
with the head positioned to expose the largest area of neck possible.
The skin, having been clipped of long hair from the mandible to the
sternum over the area of both anterior triangles, was swabbed with
70 percent ethyl alcohol for one minute making sure that the skin
and all the residual hair were well saturated. The operator and
assistant then briefly scrubbed. The instruments were removed from
the heating oven on a tray and the necessary sterile equipment was
placed near at hand. Using scissors and thumb forceps, a mid-line
incision was made over the larynx and trachea for about three inches,
and the skin margins retracted laterally exposing an area about 3
inches long and 2 inches wide. The scissors and forceps which had
been used to incise the skin were then discarded as a sterile
precaution. Employing a blunt dissecting probe, the carotid artery
on either side was exposed and isolated for one inch of its length.
A small serrefine was placed at the distal end of the exposed length

of the artery, and another at the proximal end. Then an eighteen gauge hypodermic needle on a 30 ml. "Leur-Lok" type syringe was inserted into the artery between the two serrefines and pointing towards the heart. The proximal serrefine was removed and although often unnecessary, was placed on that portion of the artery occupied by the needle so as to "tie" it in place. The syringe slowly filled by the rabbit's blood pressure without any encouragement from the operator.

The usual amount of blood taken was between 60 and 70 ml. Early in the project, 35 ml. was withdrawn followed by the operation to obtain the marrow after which the final 35 ml. was withdrawn. This practice was based on the concept that an interval is necessary for the boistering of the circulating volume by tissue fluids if 70 ml. is to be obtained. However, it was found that 70 ml. could be drawn at once with obvious advantages and this was practiced during most of the operative procedures. When the first syringe was filled, it was disconnected from the needle, handed to the assistant, and immediately replaced by a fresh syringe which began to fill. Meanwhile the assistant emptied the first syringe-full of blood into a sterile 50 ml. centrifuge tube. When the second syringe was filled, the serrefine was replaced at the proximal end of the exposed area of the artery and the needle withdrawn. This blood was introduced into another 50 ml. tube and the two were balanced and centrifuged as previously detailed. The entire procedure from the beginning of the incision required about five minutes.

When experiments with heated serum were initiated it became obvious that an anaesthetized rabbit could not survive the hour and a half necessary for the preparation and heating of the serum after having had almost one-third of its circulating volume removed. Since the post mortem autolytic processes and clotting of the circulating blood are not long delayed, it was felt that marrow obtained from a rabbit that had been dead for longer than a few minutes would be undesirable for culture. It was theorized that if the blood could be withdrawn sterily by cardiac puncture from an unanaesthetized rabbit, the rabbit would be much more likely to survive than if under the influence of depressant drugs.

The rabbit to be used was clipped of long hair over the cardiac and surrounding area, then tied and held in the same manner as described in a previous section. Sterile syringes and needles were employed throughout. The cardiac outline was approximated by palpation and the region swabbed with alcohol. The puncture was done in an identical manner to the puncture for hemorrhage except that if because of a bad approach, the needle had to be completely withdrawn, and was replaced with a fresh sterile one. As was theorized, the rabbits remained alive for the hour and half during which time the serum was being prepared.

C. Obtaining the Marrow

When the processing of the serum was complete the next procedure was begun. Before the introduction of hemorrhage to stimulate marrow hyperplasia, the marrow of both femuri was used. However, after hemorrhage, the ends of one femur provided sufficient tissue. Following a similar procedure to that employed in approaching the carotid artery an incision was made parallel to and over the femur, extending from the mid dorsal line to 2 cm. distal to the femoral-tibial articulation and the skin was retracted. With fresh instruments, the muscle groups were separated exposing the lateral surface of the femur. The soft tissue was cleaned away as well as possible with scissors and scalpel. Then grasping the shaft of the femur with a tenaculum, as close to the head as possible, the capsule of the joint and the ligamentum teres were cut and the entire femur drawn out of its bed, clearing adhering soft tissue to free it. The femoral-tibial joint was similarly disarticulated and the femur lifted out and scraped clean with a sterile scalpel.

VI. EVOLUTION OF A METHOD FOR PREPARING MARROW

    In the initial stages of the project, the marrow was taken from both femuri. Each bone was cut in half with a small high speed rotary saw, the saw-blade having been sterilized in a similar manner to the instruments. The tissue was aspirated from the marrow cavity through a modified sixteen gauge hypodermic needle into a 30 ml. "Luer-Lok" type syringe which contained about 5 ml. of balanced salt solution. The marrow-salt solution mixture was then discharged into a small sterile glass tube in which it was homogenized with a sterile rubber piston thinly coated with paraffin wax. After treatment varying from two to fifteen minutes depending on the character of the marrow, the homogenate was transferred to a 15 ml. centrifuge tube and spun for a few minutes at 1,000 r.p.m., and for about ten minutes at 2,500 r.p.m. This resulted in a layering of the contents, from the top down, fat, balanced salt solution, white cell layer, a "buffy coat" of myeloid and erythroid cells and at the bottom, mature red cells. The myeloid-erythroid and upper part of the red cell layer were transferred to sterile Wintrobe tubes and spun again, first slowly and then at a high speed. A similar though more complete layering resulted, and again the myeloid-erythroid layer was withdrawn and placed in about 10 ml. balanced salt solution and resuspended by shaking and agitating with a glass Wintrobe pipette. Following a

nucleated count, balanced salt solution and serum were added to produce a final nucleated population of about 2,000 per cubic millilitre in medium containing 33% serum. Then by sampling with a 5 ml. pipette from the master suspension, four theoretically identical cultures were produced. The entire procedure consumed about two hours.

The first modification was the introduction of an apparatus which made possible the rapid aspiration of the bone marrow from the lumen of the femur. Air was aspirated from the flask through the side arm attached to a motor vacuum. This provided a means of drawing the marrow into balanced salt solution in the flask in a continuous stream, reducing the time of aspiration from fifteen minutes to one minute.

The method of homogenization was not satisfactory for several reasons. It was very traumatic to the cells, exposed them in some degree to contact with rubber which is extremely toxic (28, 61), and most important, it failed to facilitate separation of cellular elements from fat. In regard to the latter, during several of the earlier cultures, fat was present in such large quantities, that it acted as a filter, drawing almost all of the cells out of the suspension. Hemorrhaging of the rabbits decreased the amount of fat but many cells were still being lost. Other methods of homogenizing were tried. The initial suspension of marrow was placed in an 100 ml. erlynmeyer flask with a glass stopper,

Figure 11. Equipment for the Processing of Marrow.

       1 - Marrow tubes

       2 - Homogenizers

       3 - Master suspension flask

       4 - Aspirator head

       5 - Sampling head

Figure XX. [illegible caption text]

1. [illegible]
2. [illegible]
3. [illegible]
4. [illegible]
5. [illegible]

diluted with balanced salt solution and the contents shaken vigorously for some minutes. On cessation of motion the fat rose to the surface and could be removed leaving a fat-free suspension. Several culture suspensions were prepared by squirting the initial suspension repeatedly under high pressure through a narrow glass tube. The fat rose to the surface and was removed. These methods all had the major drawback. They were traumatic and prolonged and most important, were undependable, sometimes working well, sometimes not at all, the results being more often poor than good.

Half way through the project, the use of balanced salt solution was abandoned in favour of pure serum. The small amount available made necessary the use of smaller equipment. From standard laboratory glassware, the following pieces of equipment were produced:

(1) From the standard 15 x 125 mm. test tubes, several "marrow tubes," by simply reducing the length to about 80 mm.

(2) Homogenizers - made by sealing the end of a thick glass tube and blowing a bubble.

(3) Aspirator heads - these fitted the marrow tubes and small erlynmeyer flasks and when attached to an electric vacuum source, were used for marrow aspirations and transfer of suspension from one container to another. A "Y" tube in the vacuum line served as an escape and allowed the degree of vacuum to be quickly and easily controlled.

Figure 12. The Processing of Marrow.

A. Aspiration

The escape could be closed by placing a thumb over the open arm of the "Y."

(4) Several erlynmeyer flasks, 25 ml. in size for the master suspension.

(5) A sampling head to fit the erlynmeyer flasks; used to divide the master suspension by repeated sampling into as many cultures as are desired. The homogenizers, homogenizer tubes and erlynmeyer flasks were all siliconed.

An aspirator head was attached to a marrow tube, and after 6 ml. of serum had been drawn in (so that the tube was about half full), the marrow was sucked from the ends of the femur. The aspirator head was then removed and the homogenizer applied briefly. The term "homogenizer" is actually a misnomer since the process is just a mixing of short duration. Nevertheless, the term is retained because of its analogy with the homogenizer used in the original process. Without delay, the marrow tube was covered (with a sterile rubber plug) and placed in the centrifuge where it was spun for 5 minutes at 1,500 r.p.m. At the end of this period, the contents of the tube were in several layers, the fat at the top, cellular and fibrous tissue at the bottom, and the serum between. After the fatty and serum layers had been removed and discarded, a fresh aspirator head was attached to the marrow tube which now contained a little serum, the cells, and fibrous debris, and about 6 ml. of serum were drawn in to replace that which had been discarded.

Figure 12.   The Processing of Marrow.

B.   Homogenization

Figure 12.   The Processing of Marrow.

C.   Sampling.   The rubber
tube is attached to a small bulb and
serves as the means of distributing
the marrow suspension into several
culture flasks.

The aspirator head was removed, and using a fresh homogenizer the cells were resuspended. Usually large clumps of fibrous tissue which had been spun down with the cells became evident in this resuspension. The marrow tube was allowed to sit for two minutes during which time most of the fibrous tissue settled to the bottom. Meanwhile another aspirator head was placed on an erlynmeyer flask. Using this the cell suspension was drawn from the marrow tube leaving the fibrous tissue behind. Then serum was drawn into the erlynmeyer increasing the volume of the cell suspension to about 25 ml. The aspirator head was then replaced with a sampling head, and the master suspension was divided into as many cultures as were required. To summarize briefly.

(1) Serum drawn into marrow tube.

(2) Marrow aspirated into tube.

(3) Marrow mixed with serum by the use of a glass homogenizer.

(4) Centrifuged for 5 minutes.

(5) Fat and serum drawn off.

(6) Fresh serum added.

(7) Cells resuspended with homogenizer.

(8) Fibrous material allowed to settle.

(9) Cell suspension drawn into erlynmeyer "master" flask.

(10) Serum added to produce master suspension.

(11) Master suspension divided by sampling.

During the early trials of this method, it was noticed that some clotting of the marrow occurred immediately after its aspiration. To prevent this, 50 units of heparin in solution was added to the serum in which the cells were first suspended.
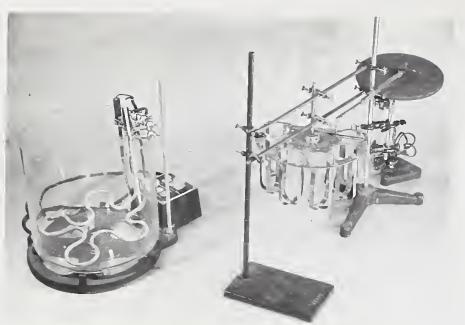
Figure 13A. Water Bath and Culture
Agitator Unassembled.
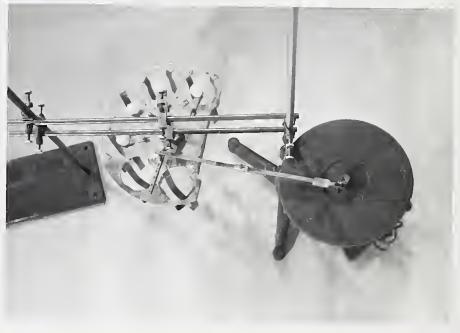
Figure 13B. Agitator from above showing
turntable and culture
basket.

Figure 13C. Culture basket with four
culture bottles.

Figure 13. Culture Agitation.

Figure 17a. Water bath and Sullivan aeration fermenter.

Figure 17b. Aspirator from water bottle, manometer and culture vessels.

Figure 17c. Aeration vessels with cotton filters.

Figure 17. Culture apparatus.

VII. THE CULTURES

A. Bottles

In the first half of the series, the bottles used for cultures were 250 ml. centrifuge bottles of special lead-free glass. Each culture was 15 ml. in volume and covered the bottom of the bottle to a depth of about one centimeter.

At the initiation of pure serum cultures, 25 ml. erlynmeyer flasks were obtained. The cultures, between 4 and 6 ml. in volume, covered the bottom to a depth of less than a centimeter.

B. Agitation

All cultures were continuously agitated with a circular back-and-forth motion at the rate of 54 cycles per minute, revolving through an arc of 35 degrees. The culture bottles were attached 4 inches from the centre of rotation. The agitator consisted of a brass disc rotating in a horizontal plane about its centre. The power was supplied by a slightly modified electric gramophone motor-turntable unit. The movement was enough to prevent the cells in the bottles from settling out, but there was no visible evidence of "sloshing" of the fluid or of centrifical force.

C. Incubation

All cultures were incubated at 37.5° C. in a standard Fisher Water-bath. The temperature was controlled by a drop of ether-acetone mixture in a mercury thermoregulator.

VIII. CELL COUNTS

It is necessary in fluid cultures to estimate the concentration of the total cells in suspension, or of any one type of cell, both at the beginning and at the end of the culture period. There have been many different methods described for counting cells in suspension, but the two most important to consider are: (1) use of a photo-electric cell, (2) the hemocytometer.

A. Equipment

The use of a photo-electric system for counting cells in bone marrow cultures was reported by Norris and Majnarich (45). However, previous workers in this laboratory (43) employed the technique with little success. In a review of the method of cell counts with photo-electric equipment, Leon Blum (2) stated that the reading is dependent on the concentration of cells in suspension and their size and hemoglobin concentration (in the case of nucleates, the nuclear characteristics are of parallel importance -- author's addition). From Blum's work it can be concluded that the variabilities in cell size, type and density, and the presence of variable amounts of debris make impossible the application of the photo-electric method to suspensions of marrow cells which have been cultured.

A. Unassembled.
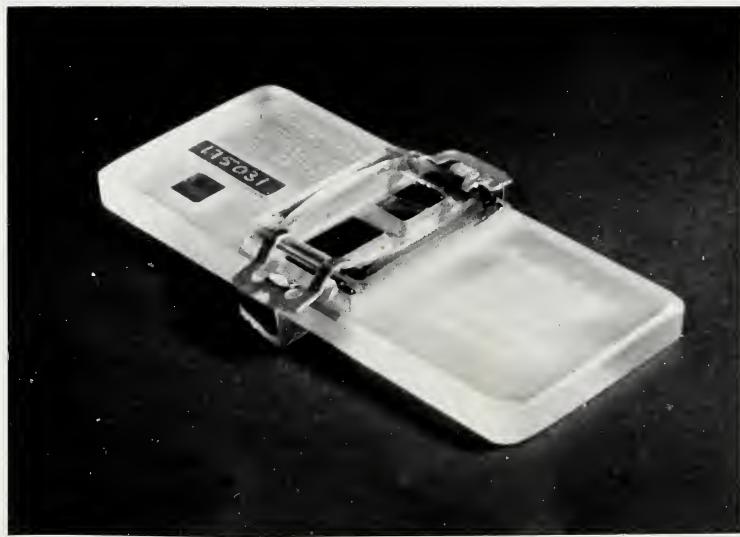
B. Assembled.

Figure 14. Hemocytometer, re-enforced coverglass and special clips.

A. [illegible caption]

B. [illegible caption]

[illegible figure caption]

The hemocytometer was utilized as the medium of
estimating cell concentration. A single American Optical Company
Spencer "Bright Line" hemocytometer was employed throughout the
entire project. A Hausser reinforced cover-glass, slightly modified,
was held in place by small German silver spring clips (84). The
plastic reinforcing border is said to prevent twisting in the cover-
glass which would result in uneven distribution. The cover-glass
was marked so that it could always be placed on the hemocytometer
in the same position. The clips held the cover-glass preventing
shifting during manipulation and more important, any tendency for
the cover-glass to lift due to fluid pressure when the chamber was
being charged. The dilutions were made using standard Thomas white
pipettes with dilution ratios of one to ten and one to twenty. The
pipettes were shaken in an Arthur H. Thomas electric Research Shaker.

B. Procedure of Cell Counts

The procedure was developed and applied in an
unchanging manner; there was no variation in technique.

Using sterile precautions, a diluting pipette was
removed from its sterile tube and attached to the rubber mouthpiece.
A sample was withdrawn from a culture bottle which had previously
been agitated with a circular motion for twenty seconds. The tip
of the pipette was wiped dry and then the sample adjusted to the
proper mark by touching the tip repeatedly to the skin of the palm
of the hand. Then the dilutent was drawn up to the second mark,

the pipette was agitated in the shaker for thirty seconds and then
placed with an attached identification on the table to be counted
at the completion of sampling. All cultures were sampled in a
similar manner before counting was started. The dilutent was
kept in a stock bottle, a little of which was poured into a vial
whenever needed. Any which remained in the vial at the completion
of sampling was discarded.

The hemocytometer was cleaned with a soft cloth and
distilled water and, occasionally, alcohol. The chamber and cover-
glass were assembled and the pipettes to be counted shaken for a
thirty second period. It was then rotated ninety degrees and shaken
for another thirty seconds. As soon as the shaker had stopped the
pipette was removed, several drops shaken out, the tip wiped and one
side of the chamber charged. The pipette was returned to the shaker
for another thirty seconds, after which several more drops were
shaken out and the other side charged. The same side of the chamber
was always charged and counted first.

C. Diluting Fluids, Stains and Counts

For some time, the procedure consisted of doing
total and nucleated counts and by subtracting, finding the red
cell concentration. The dilutent for total counts was Toisson's
fluid. The area counted was five twenty-fifths of a square milli-
meter, high power microscopy being employed. The nucleated counts

were done with 1% acetic acid as a dilutent, and crystal violet
to differentiate cells from fat globules. The area counted was
four square centimeters, the low power objective giving sufficient
magnification. It soon became apparent that in the subtraction of
two estimates, the possible error of the result is the sum of the
original errors. That is, the errors in total and nucleated counts
are combined to give a large error in the figure which represents
the red cell concentration. Therefore, a method was initiated whereby
both the nucleated and red counts could be done in the same chamber
at the same time, and over the same area. The dilutent was isotonic
saline with methylene blue. The same procedure was used in sampling
and filling the chamber as previously described. The nucleated cells
stained blue, and the non-nucleated or red cells did not stain, or
stained only slightly. Using a two-place differential counter, the
cells were counted at the same time, about four hundred cells being
counted on each side of the chamber. If the concentration of cells
was such that the count would be higher, only half the area was
examined, and the factor (that figure which when multiplied by the
actual count gives the concentration of cells per cubic mm.) was
suitably adjusted. Two pipettes were used for each culture, each
pipette being used to charge both sides of the hemocytometer.
Samples were taken and counts were done in the order A, B, C, D, D,
C, B, A; or when only two cultures were used A, B, B, A. This
resulted in four counts being obtained for each culture at a given
time.

The following addition was recommended by the Board of Examiners:

Before actual experimental cultures were undertaken, several days were spent in standardizing the counting technique and eliminating personal error. Eventually, using the same cell suspension, many successive counts could be made with the variation between them not exceeding ten percent of the average cell count. With the sum of the personal error and the normal variation reduced to below ten percent and considering that the average of four counts was used for each initial and final value during culturing, it was felt that counting error in the experimental work had been minimized.

Some difficulty was encountered in the cultures
with plasma medium. The methylene blue in the counting dilutent
has an anti heparin activity (the stain being acid and the heparin
basic) with the result that there was a tendency for fibrin to
form. Since it is the acid nature of the stain that causes its
combination with nuclear material, several acid stains were tried.
However, all except methylene blue caused an immediate obstructive
formation of the fibrin. Methylene blue, perhaps because less is
required to produce sufficient coloration, only occasionally
caused the interfering substance to appear.

D. Discussion of Errors in Counting

Variation in results can be introduced from three
sources; the personal error, the mechanical error, and normal
variation.

Personal error includes such things as inaccurate
sampling and diluting, failure to shake drops from the hemocytometer
before charging, over or undercharging and actual miscounting.
These can be minimized or eliminated by practice. The technician
or research worker who does many counts a day introduces little or
no variation into the results due to his own error*

Mechanical error stems from badly calibrated
equipment such as pipettes, hemocytometers, and cover-glass,

*See page facing page 74.

unsatisfactory mechanical agitation and loss of cells due to mechanical trauma. These errors can be minimized by using the same equipment whenever possible and by employing a standard technique. Bryan, Chastain, and Garrey (6) in a statistical study of counting methods, attributed one of the major sources of error to the failure to obtain an even suspension of cells in the pipette. Agitation, mechanical or otherwise, when in one plane only, does not give good mixing, the resultant dispersion of cells being uneven (5). A machine was devised by Bryan and Garrey (5) which rotated the pipette simultaneously around two axes, after the fashion suggested by Pontain who devised the pipette with a bead in the bulb. This is reported to have given much better results, and has subsequently been used satisfactorily in research. The agitator used in connection with the investigations reported in this thesis was one which shook the pipette in a plane at right angles to its long axis at a frequency of 2400 cycles per minute for thirty second periods.

When other sources of error are eliminated, the variation becomes dependent on the normal variation associated with the random distribution of cells in the chamber. This variation has been minimized by the counting of as many widely separated areas as possible.

E. Statistical Analysis

It is desirable, and indeed almost a necessity in this type of study to determine the standard error of analysis by a statistical survey. Since no statistical analysis has been attempted, there is some hesitation in suggesting even an approximate value with reference to the accuracy of the counts. In view of this, it may be said that absolute expressions of results cannot be submitted.

However, considering that repeated counts were made in every case and that the technique of counting was standardized and was done repeatedly by the same person, GENERAL TRENDS may be accepted as indications and that conclusions, with some reservation, may be made from these trends.

IX. HISTOLOGICAL TECHNIQUE

A. Smears

Due to somewhat unsuccessful attempts at staining, differential counts were not done. Smears by their very nature, must be thin to be effectively stained. Because of the low concentration of cells, smears were made rather thick and as a result could not be optimally stained. Wright's stain, Geimsa's and May Grünwald-Geimsa stains were used.

B. Sections

All tissues were fixed in Maximow's variant of Zenker's formaldahyde, and embedded in paraffin. The stain was Hematoxylin and Azur II Eosin in buffered solutions. An occasional section was stained with Geimsa's stain. Whole femurs were fixed for three days in Maximow-Zenker fix (penetration of the fixative was facilitated by drilling many 2 mm. holes in the shaft of the bone). Decalcification was affected with five percent formic acid for two to four days. These tissues were embedded, sectioned and stained much as described for tissues.

Photomicrographs were taken using a Leitz-Leica C3 miniature camera and a Leitz "Micro-Ibso" photomicrographic adapter. The microscope was a Bausch and Lomb. The light source was from a Mazda 3200° K photo flood lamp. The film was fine grained panatomic film, speed rating 50 ASA units.

Photographs were made of fixed sections and smears in the usual
manner with all powers. Pictures made of cells in the hemocytometer
were made under high power or high power with the microscope tube
extended. In addition some photographs were taken of cells in
suspension (as in the counting chamber) in a special chamber which
allowed the use of oil immersion.
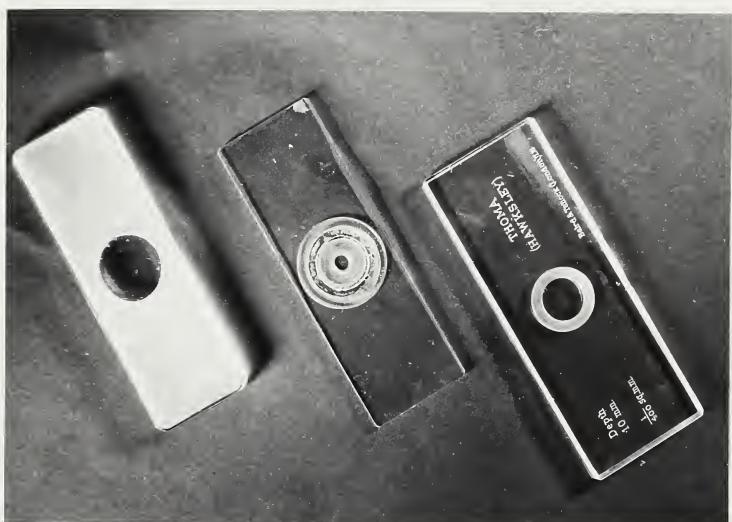
Figure 15.   Microculture Chambers.

Left - Hanging drop slide.

Centre - Modified MacNeil slide
        for lying drops.

Right - Old style hemocytometer
        for lying drops.

Figure 19. Compensation Comparator.

Left - Standard Area Gates.

Center - Multiple Profile Gates
on Lower Scope.

Right - All Angle Comparator
on Upper Scope.

X. MICROCULTURE METHODS

Serious consideration was given to the possibility
of carrying out microcultures. An attempt was made to copy Plum's
microchamber (67). Plum's apparatus consists of a shallow compart-
ment the floor of which is a semi-permeable membrane. Below the
floor is a constantly changing nutritive, incubative (warm),
oxygenated fluid which supplies warmth, nutrition and proper gas
tension and removes metabolites and carbon dioxide. Technical
difficulties in assembling and charging this chamber were dis-
couraging.

A thermostatically controlled microscope warm stage
was designed to fit on the stage of the standard microscope and
allow direct, continuous microscopic observation of preparations
within. Two general types of chambers were introduced for use with
the warm stage; (1) the hanging drop, (2) the lying drop.

1. A small drop of marrow suspension was placed in the
centre of a sterile coverslip and spread out so as to be thin. A
tiny portion of a drop on each corner of the coverslip served as
a seal. When a hollow ground slide was inverted over the slip,
the four corner drops travelled along the periphery of the slip
sealing it to the slide producing a hanging drop preparation similar
to the one used by Emmel in the study of embryonic blood (22). This
method was tried on several occasions. Although the preparations

were not incubated, the seal consisting only of four droplets of
serum, remained intact for 24 hours with no evidence of drying out
or cell crenation.

2. The other type of chamber facilitates the main-
taining of lying drop preparations. A modified MacNeil chamber (41)
and an old style hemocytometer were considered. Both chambers
consist of a round depression in a slide with a round island in
the centre, the island being a fraction of a millimeter lower than
the surrounding shoulder. When a coverslip is placed over, a very
thin space is left over the island. A tiny drop of cell suspension
was deposited on the island and the coverslip placed over and sealed
around the edges. The suspending fluid occupies the space between
the island and the coverslip, and the cells settle onto the surface
of the island. Several of these preparations were maintained
unincubated for 24 hours without drying out.

RESULTS

In the experimental work, the marrows of 53 rabbits were studied. The results are drawn from 88 cultures on 23 marrows. (Eighty-eight additional cultures were carried out but their results are not utilized due to various reasons, notably, possible count inaccuracies, contamination, and unfavourable hypoplastic marrow.)

The numerical results are expressed in two ways.

1. The initial counts are arbitrarily called 100 % and the final counts are related to the initial count as a percentage either higher or lower than one hundred depending

on whether there was an increase or decrease in cell population.
This expresses the change in culture of the total, nucleated
and non-nucleated cell* population. It has the advantages of
being easily graphed and easily interpreted. However, it does
not express the absolute production of non-nucleated cells as
a fraction of the cells from which they arise but rather as a
fraction of the inactive and variable initial erythrocyte con-
centration.

      2. The most valuable manner of expressing erythro-
cyte production is by relating the increase in red cells to the
nucleated erythroid cells in culture. The use of this method,
however, requires that differential counts be done on stained
smears of the marrow suspensions. Because this is a time con-
suming procedure requiring special training and long experience,
the change in non-nucleate population is related to the nucleated
population as a whole. While this is not strictly accurate,
because of the possible variation in the ratio of erythroid and
myeloid elements between marrows, the use of healthy rabbits and
the averaging of the results of many cultures provides figures
which are a reasonable basis for indicating favourable and un-
favourable conditions. The erythropoietic activity is expressed

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*The terms "erythrocyte", "non-nucleated cell"
and "red cell" are used interchangeably. All refer to the
hemoglobin-containing biconcaval disc-shaped cell of the blood.
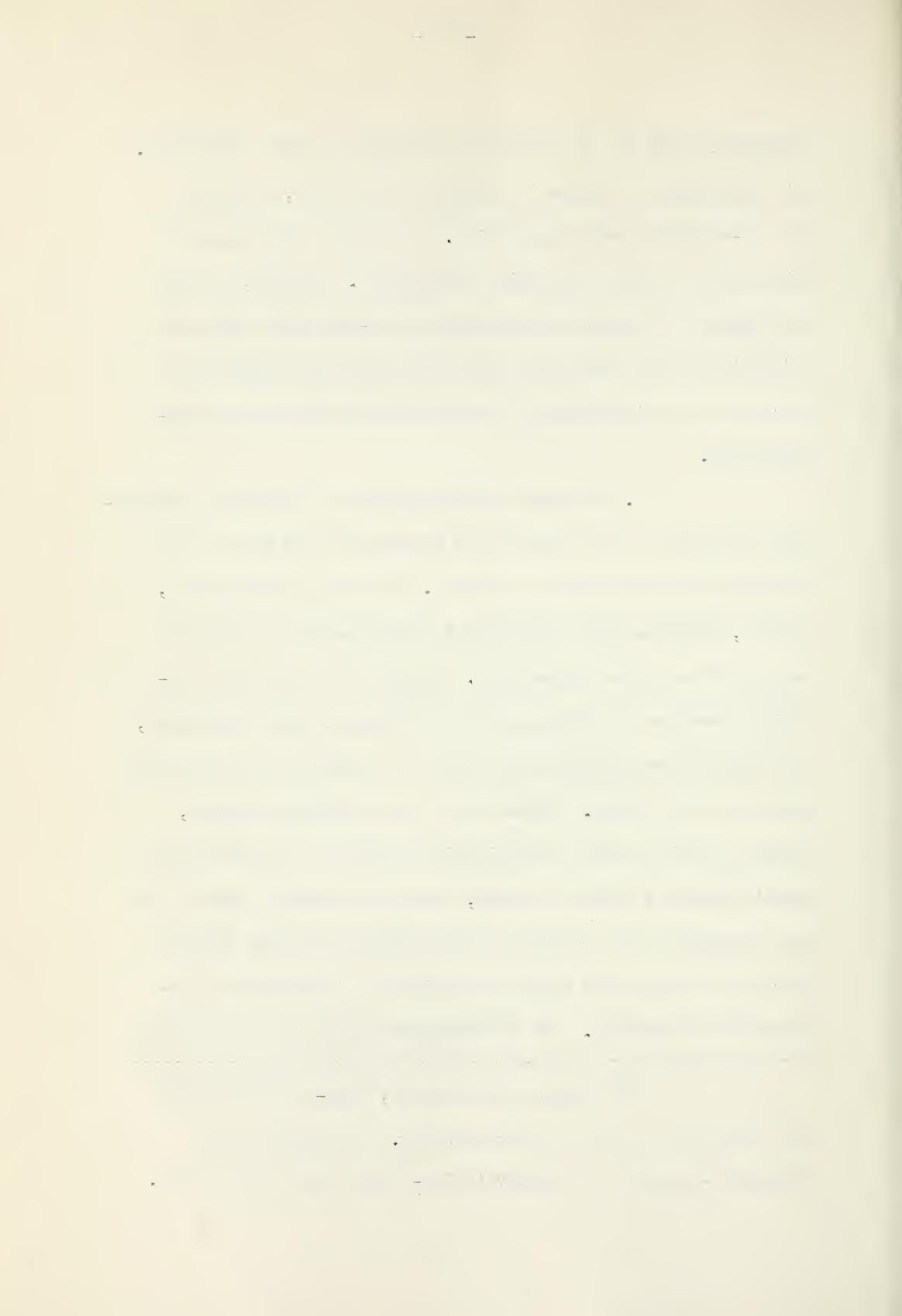
as the number of erythrocytes produced per one hundred nucleated cells, the figure for the nucleates being the average of the concentrations at the beginning and end of the culture periods.

The actual counts made at the time of culture and the various calculations derived from them are found in Appendices I and II.

I. CULTURES UNDER VARIOUS CONDITIONS

During the experimental procedures connected with this thesis, rabbit marrow was cultured under various conditions in media of various composition.

1. Balanced Salt Solution and Serum Media - "Traumatic"

The bone marrow was prepared in sterile balanced salt solution by a prolonged mechanical homogenization and two high speed centrifugings. Cells were cultured in balanced salt solution and native serum in the ratio of two parts to one.

2. Balanced Salt Solution and Serum Medium - "Atraumatic"

A preparation was utilized in which all high speed centrifuging was eliminated and other traumatic processing minimized. The marrow was briefly homogenized, diluted with balanced salt and shaken or agitated briefly, and the fat removed with a slow spin only. The culture medium was balanced salt solution and native serum.

3. Native Serum Medium

Marrow suspensions were cultured in pure native serum, the procedure for preparation being brief and atraumatic.
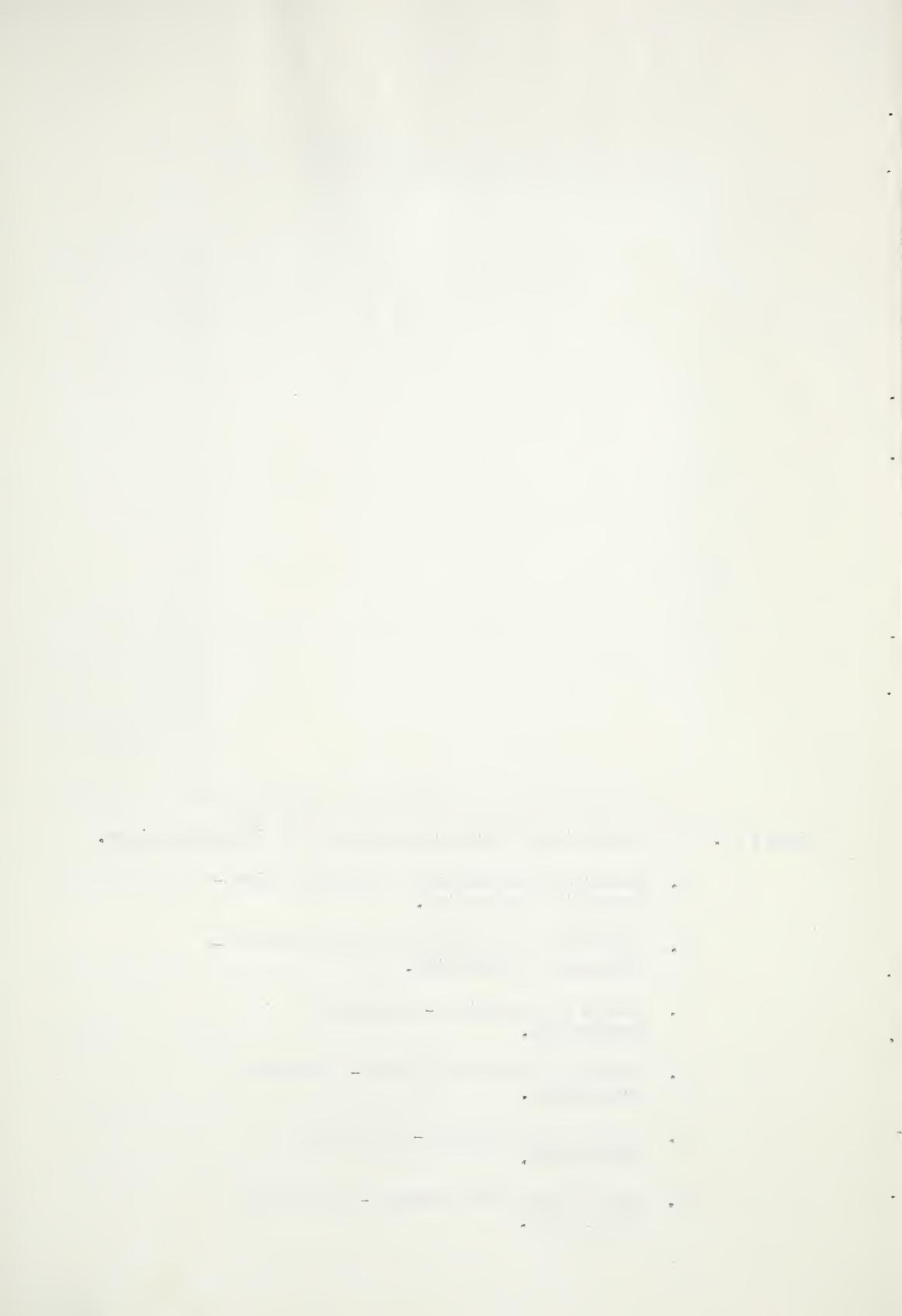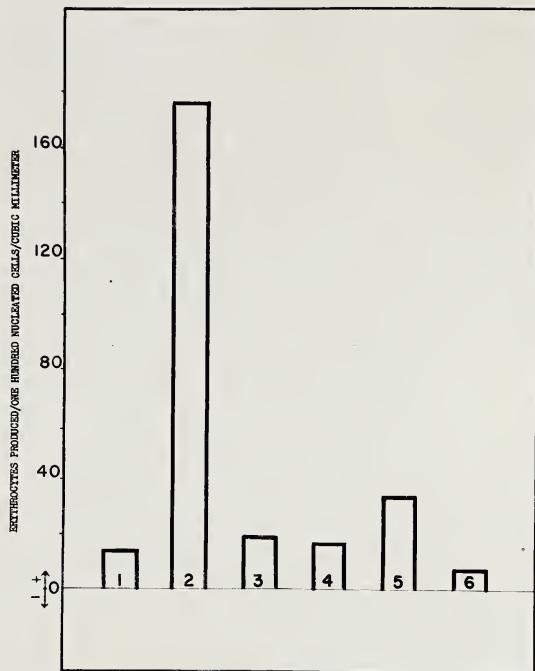
Figure 16.   The Production of Erythrocytes Under Various Conditions.

1.   Balanced salt solution and serum medium -
     traumatic preparation.

2.   Balanced salt solution and serum medium -
     atraumatic preparation.

3.   Native serum medium - atraumatic
     preparation.

4.   Heated native serum medium - atraumatic
     preparation.

5.   Native plasma medium - atraumatic
     preparation.

6.   Normal human serum medium - atraumatic
     preparation.

4. Heated Native Serum Medium

Native serum which had been heated to 56.6° C. for one hour was used as the culture medium.

5. Native Plasma Medium

Marrow suspensions were cultured in native plasma (heparinized).

6. Heated Human Serum Medium

The culture medium was pooled normal human serum which had been heated to 56.6° C. for one hour, and then treated with aliquotes of washed rabbit erythrocytes as outlined in the method. The preparation of the cultures in all cases except 1. was similarly atraumatic, with no high speed centrifugings or mechanical homogenization. The cultures in 1. and 2. were prepared in balanced salt solution and the remainder in pure native serum.

The results of the studies in the six groups mentioned are summarized in two graphs. Fig. 16 compares the changes of erythrocyte population per one hundred nucleates during the four hour period. The area above the base-line indicates increase, the area beneath the base-line indicates decrease. The production of erythrocytes is seen to be
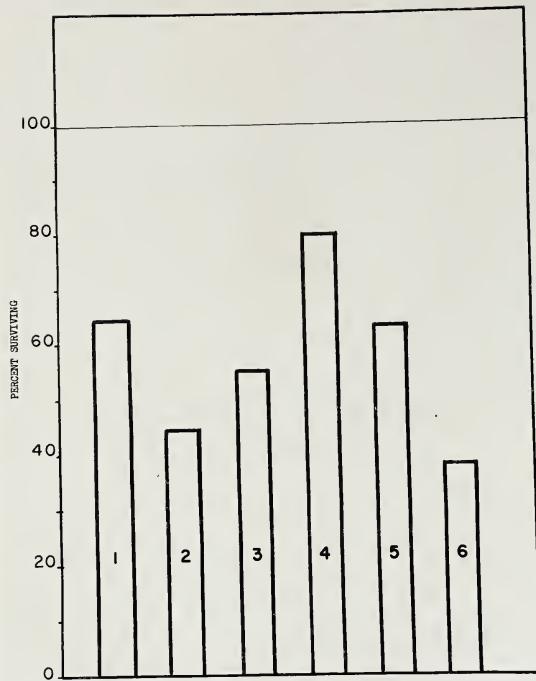
Figure 17. The Survival of Nucleate Cells Under Various Conditions.

1. Balanced salt solution and serum medium --
   traumatic preparation.

2. Balanced salt solution and serum medium -
   atraumatic preparation.

3. Native serum medium - atraumatic
   preparation.

4. Heated native serum medium - atraumatic
   preparation.

5. Native plasma medium - atraumatic
   preparation.

6. Normal human serum medium - atraumatic
   preparation.

Figure [illegible]. The Survival of [illegible] Cells Under Various Conditions.

1. [illegible]
2. [illegible]
3. [illegible]
4. [illegible]
5. [illegible]
6. [illegible]

relatively intense when the culture medium consisted of one part homologous serum and two parts Gey's balanced salt solution and when prepared without traumatic centrifugings. Plasma supports erythrocyte proliferation to a much greater degree than does serum which in turn exhibits no difference to heated serum in its ability to support erythropoiesis. Human serum was far inferior to any of the other media tried. (The addition of heparin gave better results; this is demonstrated in a subsequent section concerning the effect of heparin in the culture medium.)

Fig. 17 compares the survival of nucleated marrow cells prepared and cultured under various conditions. The survival of nucleated marrow cells was greatest in the cultures in heated homologous serum medium. In most other media the nucleated population dropped to around 50% during the culture period. In cultures using homologous plasma as a medium, 63% of the nucleates survived after four hours. The nucleat population in cultures with human serum dropped to 43%. (The addition of heparin maintained the nucleated count at 94%. This is also dealt with in a subsequent portion of the results.)
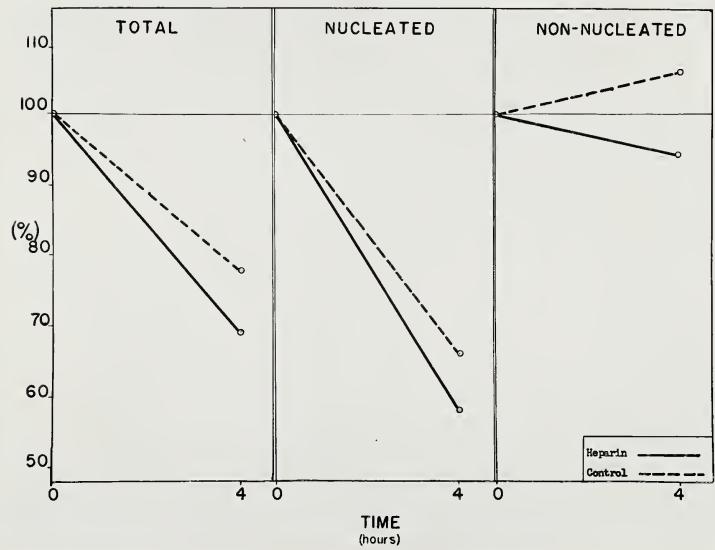
Figure 18. The Effect of Heparin.

Native serum medium, heparin
concentration = 10μ/ml. of culture.

Figure 86. The Curve of Pumping.

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II. THE EFFECT OF HEPARIN

Since the study of bone marrow "in-vitro" is
closely involved with peripheral blood characteristics such as
clotting, it is necessarily important to investigate the effect
upon the marrow cells of some potent anticoagulent. Heparin was
used in preference to the chemical anticoagulents, both because
it is more active and because evidence indicates that it is less
toxic to living cells (72).

To each of eight cultures, 0.05 ml. of heparin
solution (Connaught Laboratories - 1000 international units/ml.)
was added, providing a final concentration of ten international
units/ml. of cell suspension. As controls, eight cultures were
prepared and incubated in similar fashion but received no heparin.
Four of the controls and four of the cultures which received
heparin were suspensions of rabbit marrow cells in native serum.
The remaining eight cultures were rabbit cells in heated human
serum. The results are expressed in graph form indicating the
changes in total, nucleated, and non-nucleated cell population

A. Native Serum Medium

Fig. 18 shows the average of the results of eight
cultures of rabbit marrow in homologous serum. It can be seen
that in the cultures to which heparin was added, the red cell
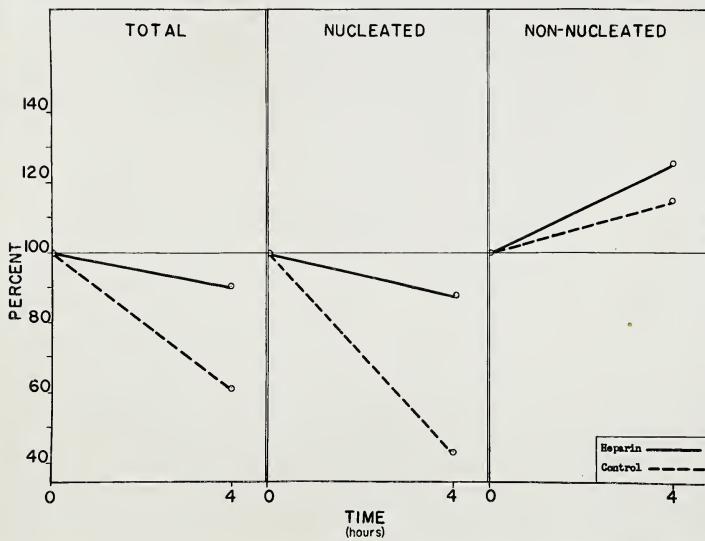
Figure 19. The Effect of Heparin.

Normal human serum medium,
heparin concentration = 10μ/ml. of culture.

Figure 1. Dry Matter of Rations.

Percent [illegible] versus Rations.
[illegible]

population dropped, while in the controls, there was a slight increase in red cells. The drop in nucleated cells was slightly more extreme where heparin had been added.

B. Heated Human Serum Medium

Fig. 19 shows the results of eight cultures of rabbit marrow in heated human serum. The erythrocyte populations of the cultures which had received heparin rose even in excess to the controls. The nucleated cells survived to a far greater degree in the cultures which had received heparin.

It is of interest to note that while the addition of heparin to rabbit marrow cultured in human serum improved both the erythropoietic activity and the nucleated cell survival, similar addition to marrow cultured in homologous serum caused a decrease in both erythropoietic activity and nucleated cell survival.
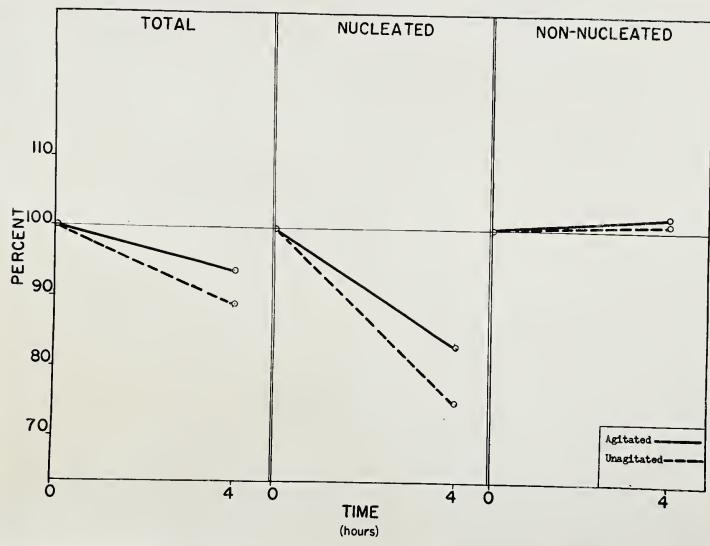
Figure 20. The Effect of Agitation.

Figure 38. The effect of motivation.

III.   THE EFFECT OF AGITATING CULTURES

It was felt that some gentle form of agitation of
the cultures during incubation would serve to keep the cells in
constant suspension, eliminating the possibility of their settling
out and coming into prolonged contact with the glass surface.
The aeration of the culture medium would be improved by the ever
changing exposed surface. The slight motion would cause a movement
of the cells within the medium thereby preventing the accumulation
of metabolites and depletion of nourishment in close proximity to
the individual cells. It was also thought that the agitation
might prevent the clumping of the cells, especially the nucleates.

Six cultures from two marrows were incubated under
identical conditions, three being agitated in the manner described
on page 69 and three were fixed without any motion.

Fig. 20 compares the average total, nucleated and
non-nucleated cell concentrations in cultures agitated and unagitated
during the incubation period. There is no notable difference between
the two other than a slightly higher nucleated cell survival in the
agitated cultures. However, agitation was helpful in that it
prevented the settling of cells and clumping, all of which will be
dealt with in a subsequent section.
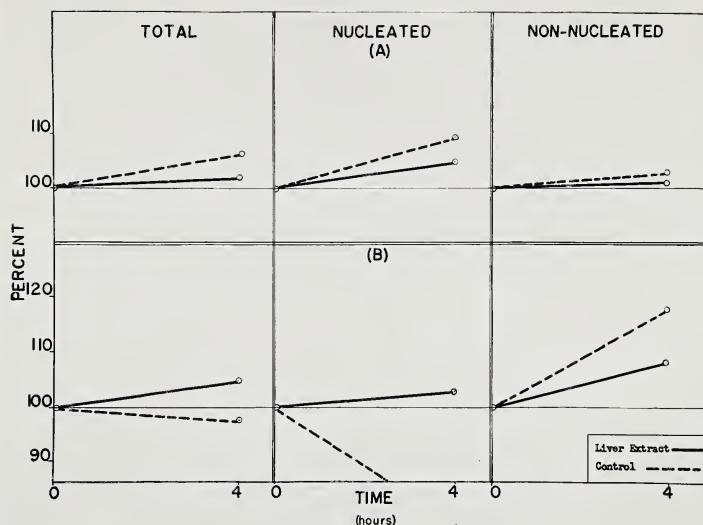
Figure 21. A. The Effect of Liver Extract.

Concentration of liver extract = 0.06μ/ml. of culture.

Figure 22. B. The Effect of Liver Extract.

Concentration of liver extract = 0.25μ/ml. of culture.

IV. THE EFFECT OF LIVER EXTRACT

Figure ... ... The Effect of Grain Refinement,
... ... Supersaturation of Trace Element,
Location of Solidus.

Figure ... ... The Effect of Grain Refinement,
... ... Supersaturation of Trace Element,
Location of Solidus.

IV. THE EFFECT OF LIVER EXTRACT

In controlled experiments, liver extract was
added to cultures which were incubated in parallel to cultures
which had received no extract. Fig. 21 shows the average of the
results of two cultures each of which received liver extract
producing a final concentration of 0.06μ/ml. of culture compared
to two cultures which received no extract. There are no notable
differences.

Fig. 22 compares the results from two cultures,
one of which received liver extract producing a final concentration
of 0.25μ/ml. of culture, and the other receiving no addition. The
erythrocyte population is lower and the nucleated cells have
survived to a greater degree in the culture which had received
liver extract.
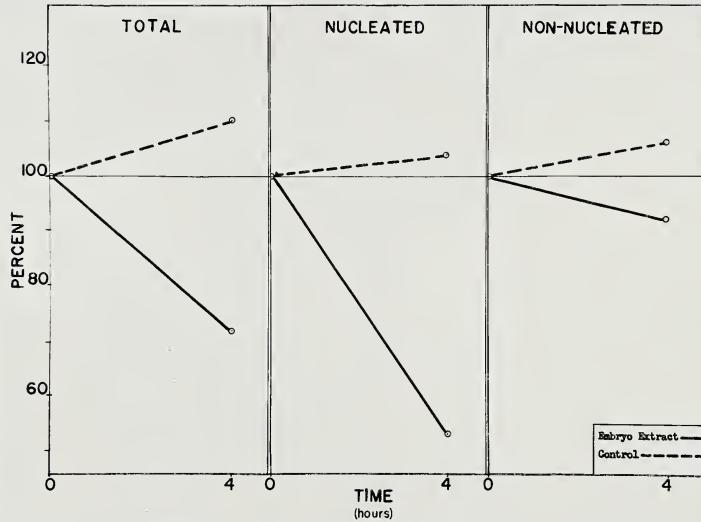
Figure 23. The Effect of Embryo Extract.

Concentration = 0.05 ml. embryo
juice/ml. of culture.

Figure 7.—The Effect of Oxygen Partial...

...Concentration...
...

V.   THE EFFECT OF EMBRYO EXTRACT

The use of embryo extract as a source of growth
promoting substances for tissue culturing "in-vitro" has long
been classical. It was felt, however, that in short term cultures,
no such substance should be necessary. Nevertheless, a short
investigation was done to determine if possible the effect on
bone marrow cells "in-vitro."

Fig. 23 compares the results of two cultures,
one of which received enough cell-free juice from the homogenate
of an eight-day chick embryo to produce a concentration of 0.05
ml. per ml. of cell suspension. In the culture which had
received embryo extract, the total, nucleated and non-nucleated
populations were depressed far below the levels in the control.
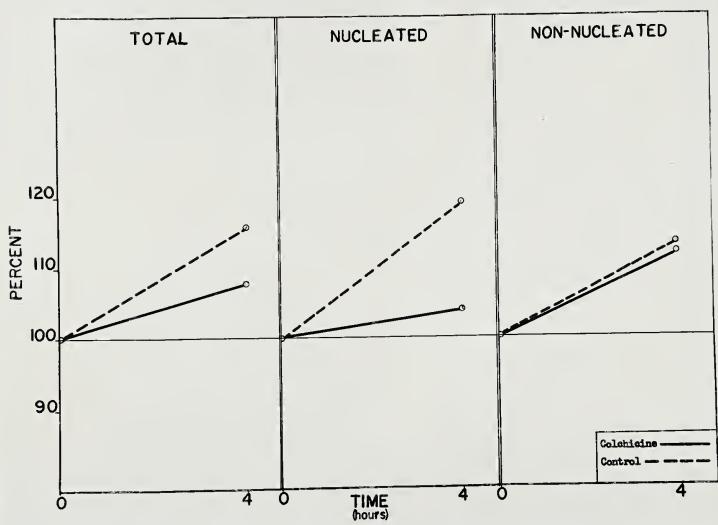
Figure 24.   The Effect of Colchicine.

            Concentration of
colchicine = 1/125,000.

Figure 94. The Effect of Temperature.

Concentration HC
Sulphuric = 2.028;0.89

VI. THE EFFECT OF COLCHICINE

A solution of colchicine* in sterile isotonic sodium chloride was made up and 0.05 ml. of this was added to a culture giving a final concentration of 1/125,000. A second culture received no addition, and acted as a control.

Fig. 24 compares the results obtained in the two cultures. Although the increase of nucleated cells is only slight in the culture containing colchicine as compared to the control, the red cell population increased identically in both.

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*An alkaloid which prevents cell multiplication by inhibiting mitosis at the metaphase.
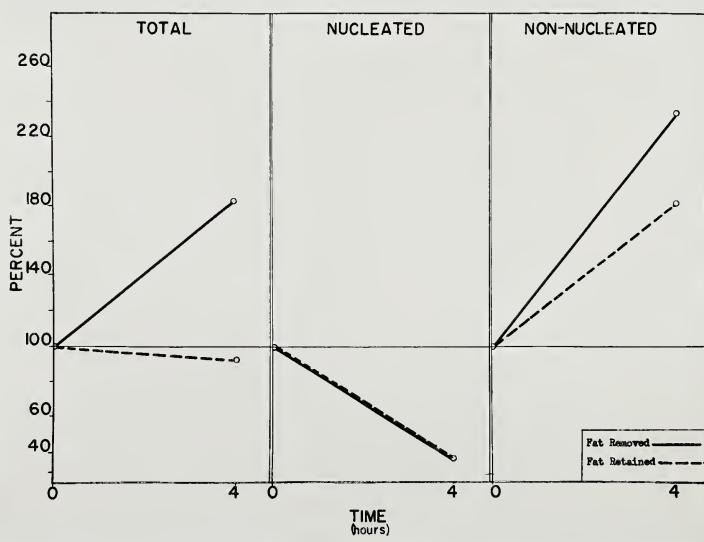
Figure 25. The Effect of Fat Removal.

VII. THE EFFECT ON FAT SYNTHESIS

One of the major difficulties associated with the preparation of serums for reduction is the presence of fat, especially in large quantities. If the cultures could be prepared successfully without removing the fat, a great deal of time and expense would be eliminated from the procedure. In addition it has been discovered that certain constituents of serums fat are of spectrophotometric importance.

Four cultures were made up from the same serums. Two of the cultures consisted of cell suspensions from which most of the fat had been removed; as a rule, centrifuging sufficing. In the other two cultures, no effort was made to remove the fat.

Fig. 17 indicates the results. Although the analyses will reproduce the fractionated, they occurred to the same degree in both the control and experimental cultures. Spectrophotometric activity in the "fattiest" cultures was much more intense than in the controls.

Graph III. The Effect of the Positive

VII. THE EFFECT OF FAT REMOVAL

One of the major difficulties associated with
the preparation of marrow for culturing is the presence of fat,
sometimes in large quantities. If the cultures could be prepared
successfully without removing the fat, a great deal of time and
trauma could be eliminated from the procedure. In addition it
has been theorized that certain constituents of marrow fat act
as erythropoietic stimulents.

Four cultures were made up from the same marrow.
Two of the cultures consisted of cell suspensions from which most
of the fat had been removed by a slow, atraumatic centrifuging,
in the other two cultures, no effort was made to remove the fat.

Fig. 25 indicates the results. Although the
nucleated cell population was fractionated, this occurred to
the same degree in both the control and experimental cultures.
Erythropoietic activity in the "defatted" cultures was much more
intense than in the controls.
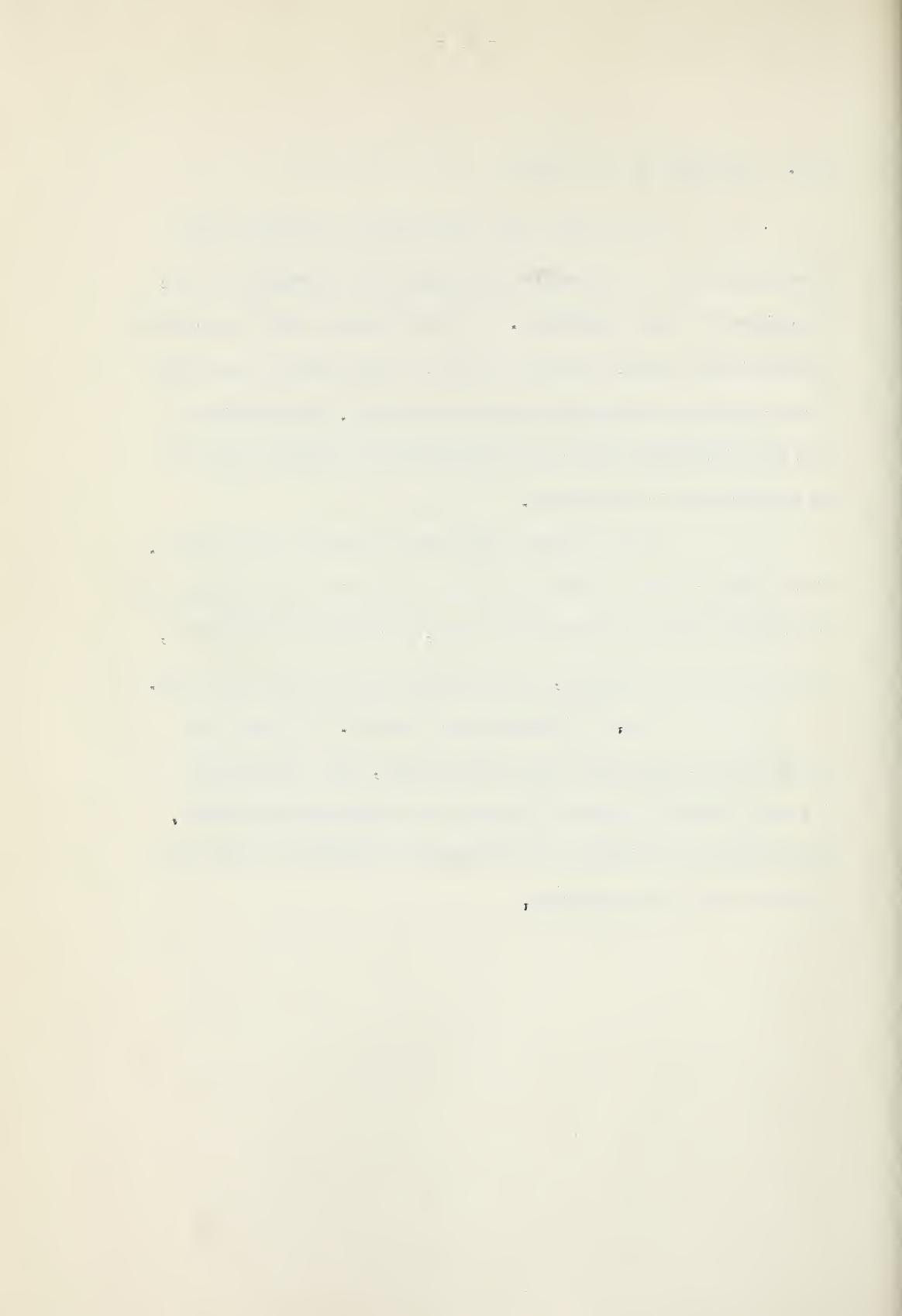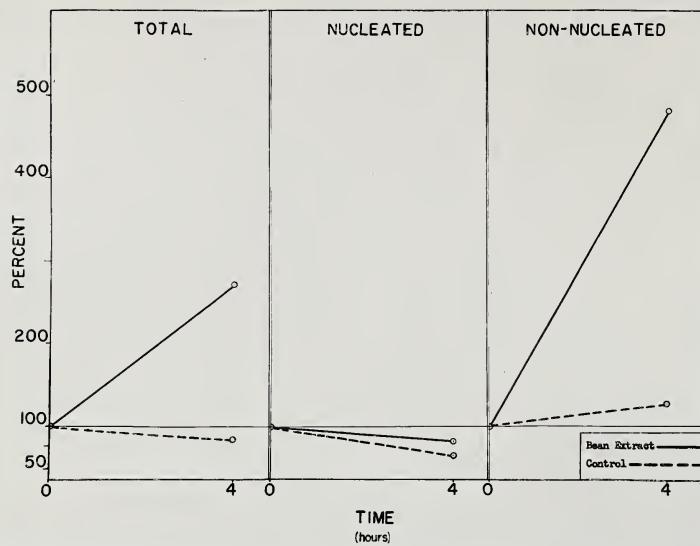
A. Four hours incubation.
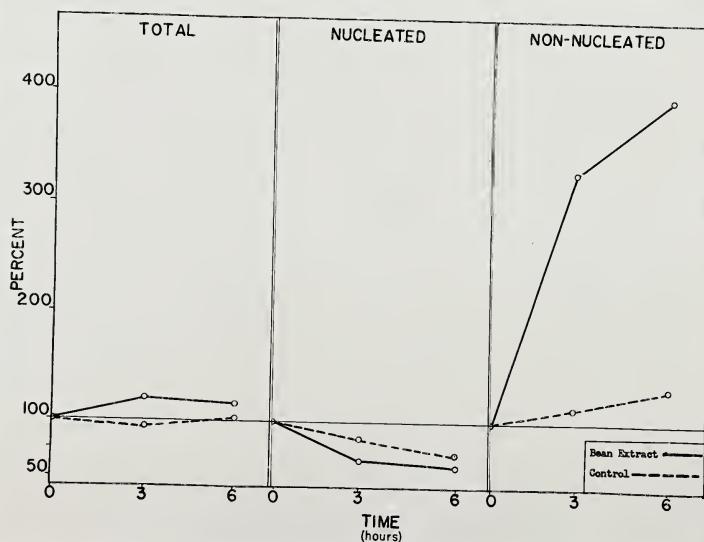
B. Six hours incubation

Figure 26. The Use of Phytohemagglutinin.

VIII. THE USE OF PHYTOHEMAGGLUTININ

It was felt that if the red cell population could be reduced to almost zero before the initial counts were done, any subsequent proliferation during the incubation period, no matter how slight, would not be masked by a relatively large number of inert cells. Moreover, it has been suggested that the control of erythropoietic activity depends upon the concentration of mature red blood cells. Therefore an effort was made to reduce the red cell concentration during the preparation of the culture using phytohemagglutinin from the common navy bean (see Appendix III for preliminary work with bean extract).

The marrow to be used was suspended and divided into two parts. Half, to act as the control, was treated by high speed centrifuging following the procedure of Nelson (43). The other half received a calculated amount of bean extract and after mixing, was centrifuged at 500 r.p.m. for 90 seconds and the resultant supernatant cultured. Fig. 26A summarizes the results of eight control and experimental cultures which were incubated for four hours. It can be seen that the erythropoietic activity in those cultures which received bean extract was far above that in the controls. Similarly the nucleated counts indicate that survival of nucleated cells is greater in the cultures which received bean extract.

Fig. 26B shows the averages for six control and six experimental cultures which were incubated for six hours, counts being done before incubation and at three hours, and six hours. Here again, erythropoiesis is evidently much higher in the cultures which received bean extract. At the end of three hours the erythrocyte population in cultures which had received bean extract had tripled. The subsequent three hour period showed only a slight rise. Erythropoiesis in the controls although much less extreme, was of the same magnitude in both three hour periods.
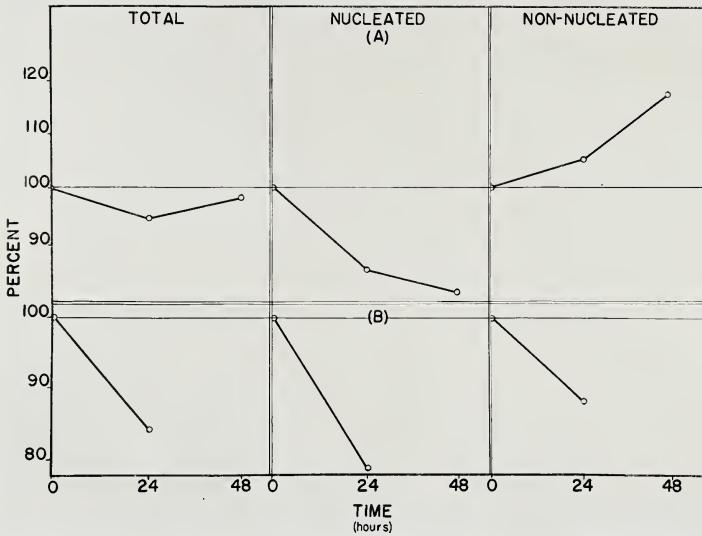
Figure 27. The Survival of Marrow Cells in Native Serum Medium

Under Refrigerated Conditions.

Temperature = 4° C.
A - Series I
B - Series II

Figure 57. The Content of Nitrte Cells in Ostion septa Forming
under metamorphose conditions.

Temperature = 5° C;
1 = Series 1
2 = Series II
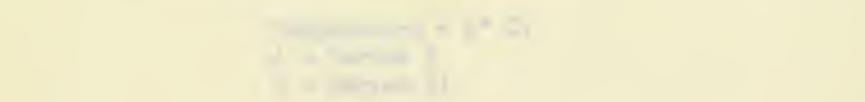
IX. STORAGE OF MARROW CELLS BY REFRIGERATION

The successful storage at low temperatures would make possible the simultaneous preparation of a large number of marrow cultures to be used as the need arises. This would also contribute towards the most desirable "tissue pool" where a large amount of marrow cell suspension could be stored indefinitely and many identical cultures drawn from it over a long period of time. The problem of refrigerated storage was subdivided into two questions. A. What is the effect of storage at low temperatures upon nucleated and non-nucleated cells in suspension? B. After refrigeration for extended periods, are the cultures able to produce red blood cells and nucleated cells during incubation?

A. The Effect on Cells of Storage at 4° C.

Twelve cultures were prepared. Four were selected at random, sampled and counted and the other eight were refrigerated at 4° C. After 24 hours, four more were selected at random, and slowly brought to 37° C., and then sampled and counted. The last four were similarly counted after 48 hours in storage.

Fig. 27A summarizes the results. There is a slight continuous drop in the nucleated cell population. The erythrocyte population increased after the first 24 hour period, and further increased during the second period.
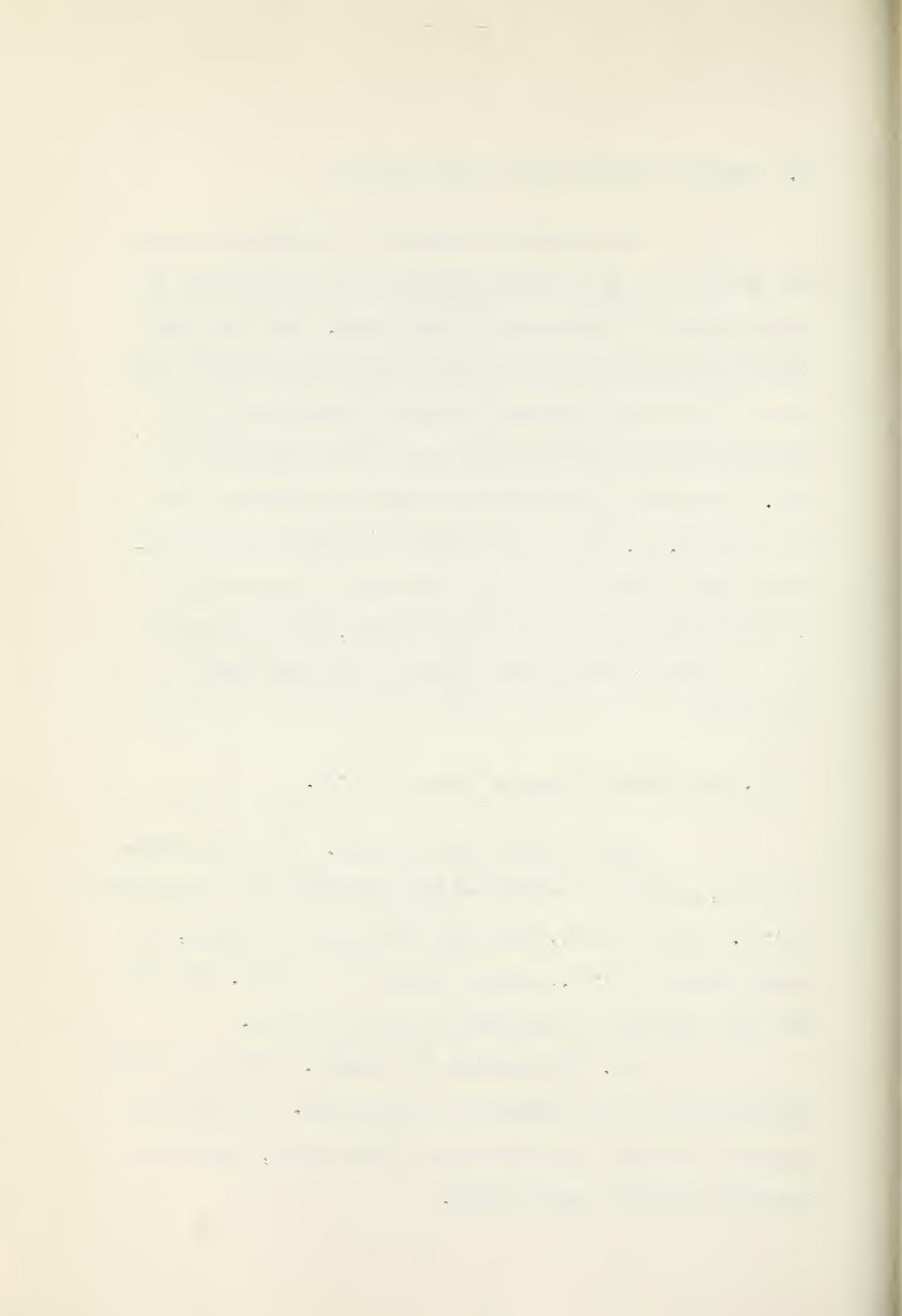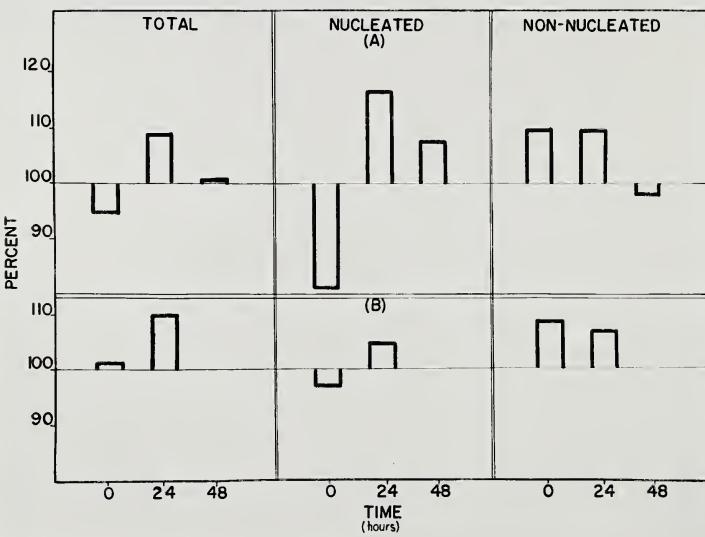
Figure 28. Hematopoiesis in Cultures of Rabbit Marrow Cells in
Native Serum Medium after Various periods of
Refrigeration at a Temperature of 4° C.

A – Series I
B – Series II

Figure XX. Comparison in Millions of Total Nerve Cells in Rat's Brain Before and After Various periods of Centrifugation at a Resultant of 5° G.

1 = Region I
2 = Region II

Twelve additional cultures from three marrows were prepared. Half were counted initially and half were stored for 24 hours before counting. Fig. 27B indicates the results. In this series, the nucleated count after 24 hours was only 78% of the count at zero hours. The erythrocyte concentration contrary to the first series, had decreased.

B. The Ability of Stored Cells to Grow

Six cultures were prepared. Two were selected at random and incubated for four hours. The remaining four were stored at 4° C. After 24 hours in storage two more cultures were incubated and after 48 hours the last two. Fig. 28A summarizes the results. There was an increase in the nucleated cell population in cultures which had been stored for 24 hours, and a similar though less intense rise in cultures which had been stored for 48 hours. Cultures which had not been stored showed a drop in nucleated cell concentration. Erythropoietic activity was identical in both the unstored cultures and those stored for 24 hours. However, there was a drop in population in those cultures which had been stored 48 hours. The results of a second series of six cultures half of which were incubated immediately after preparation and half after refrigerated storage for 24 hours are summarized in Fig. 28B. The erythropoietic activity in stored and unstored cultures was identical. The nucleated cell concentration which decreased in unstored cultures, increased in cultures which had been stored.
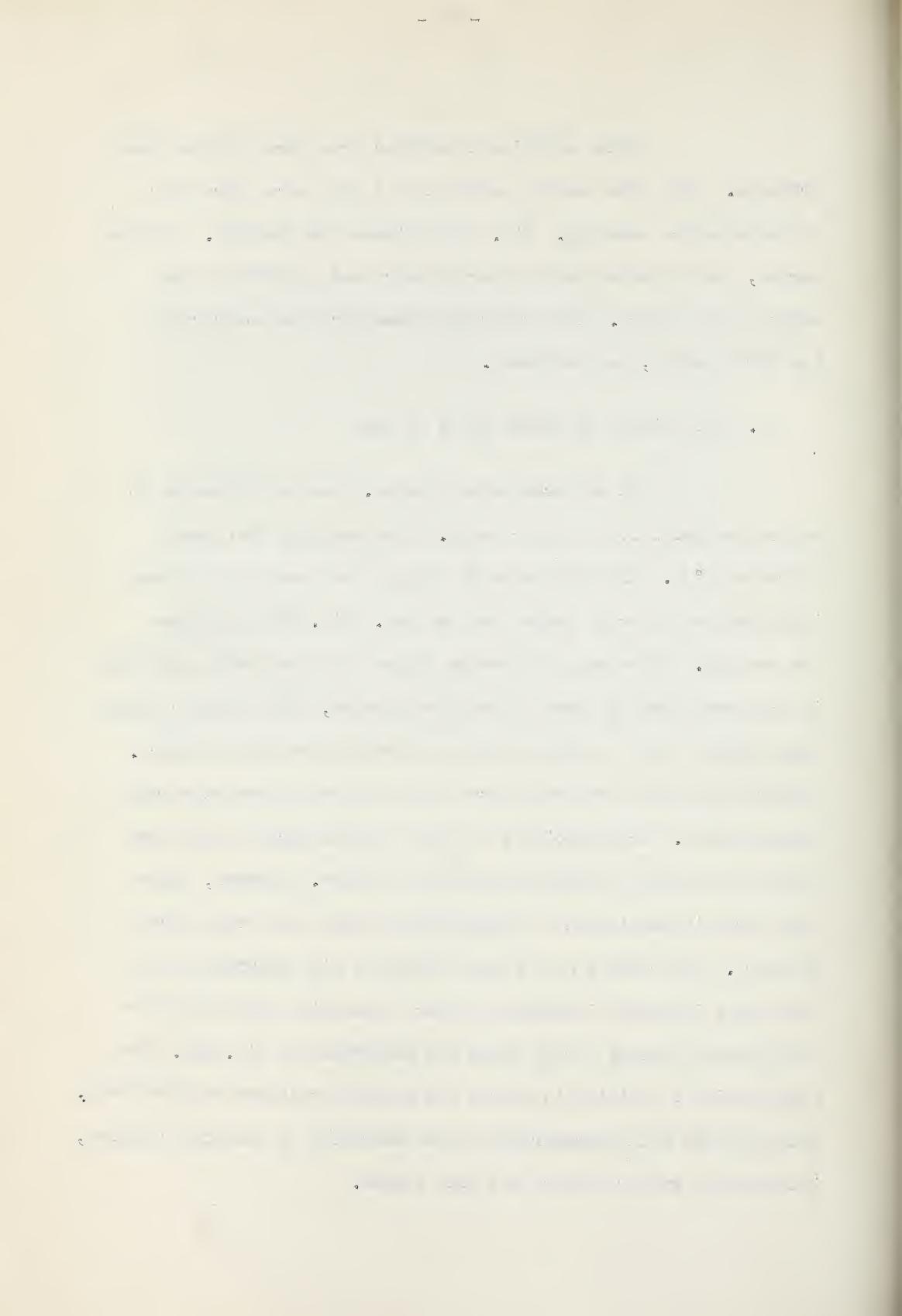
X. ERYTHROPOIESIS BY BUDDING AND CYTOPLASMIC CONSTRICTION

During the experimental work associated with
this thesis, the conflicting theories concerning the final stage
of maturation of the erythrocyte were considered. It was planned
to culture bone marrow cells in microculture chambers and directly
observe the manner in which the normoblast loses its nucleus.
Although, for technical reasons, this plan was not completed,
many observations were made in the counting chamber and in smears
of the culture suspension which support the budding theory.

A. Poikylocytosis and Spherocytosis and Anisocytosis

The cells observed in the counting chamber during
the initial counts were of approximately uniform size and shape
and appearing as biconcave discs. After the period of incubation,
many cells were irregular in shape, most often showing long, drawn
out appendages (as in Fig. 7 - from Emmel). Many cells appeared
to be spherocytes, perfectly round with no trace of the classical
biconcave form and there was greater than usual variation in cell
size during the final count.

B. Polymicrocytosis

Often in the final counts a group of nucleated
cells would be seen clumped together, and clinging to the perimeter
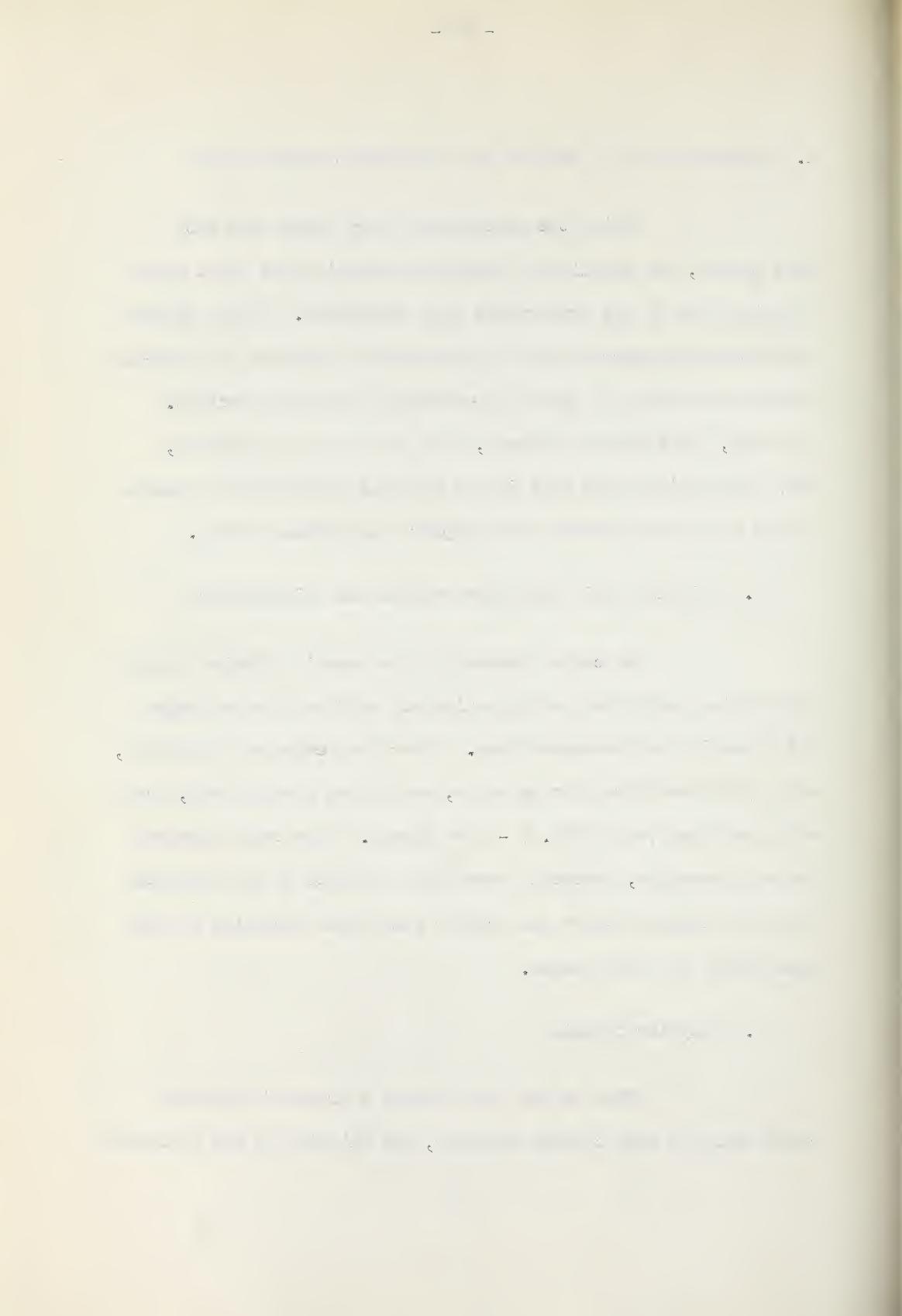
Figure 29. Budding.

Photomicrographs of cells observed in the counting chamber.

of the clump would be many round, sometimes irregular cells
approximately 4 to 6 microns in diameter and having staining
qualities similar to red cells.

C. Budding

Frequently observed in the hemocytometer, more
often during final counts were formations which are possibly
stages in budding. They consisted of what appear to be a
normoblast with a dense nucleus and light cytoplasm from which
extended a "bud" of very light, clear cytoplasm.. The bud in some
cases, was so small as to be just barely definable and in other
cases, as large as the mother cell (Fig. 29).
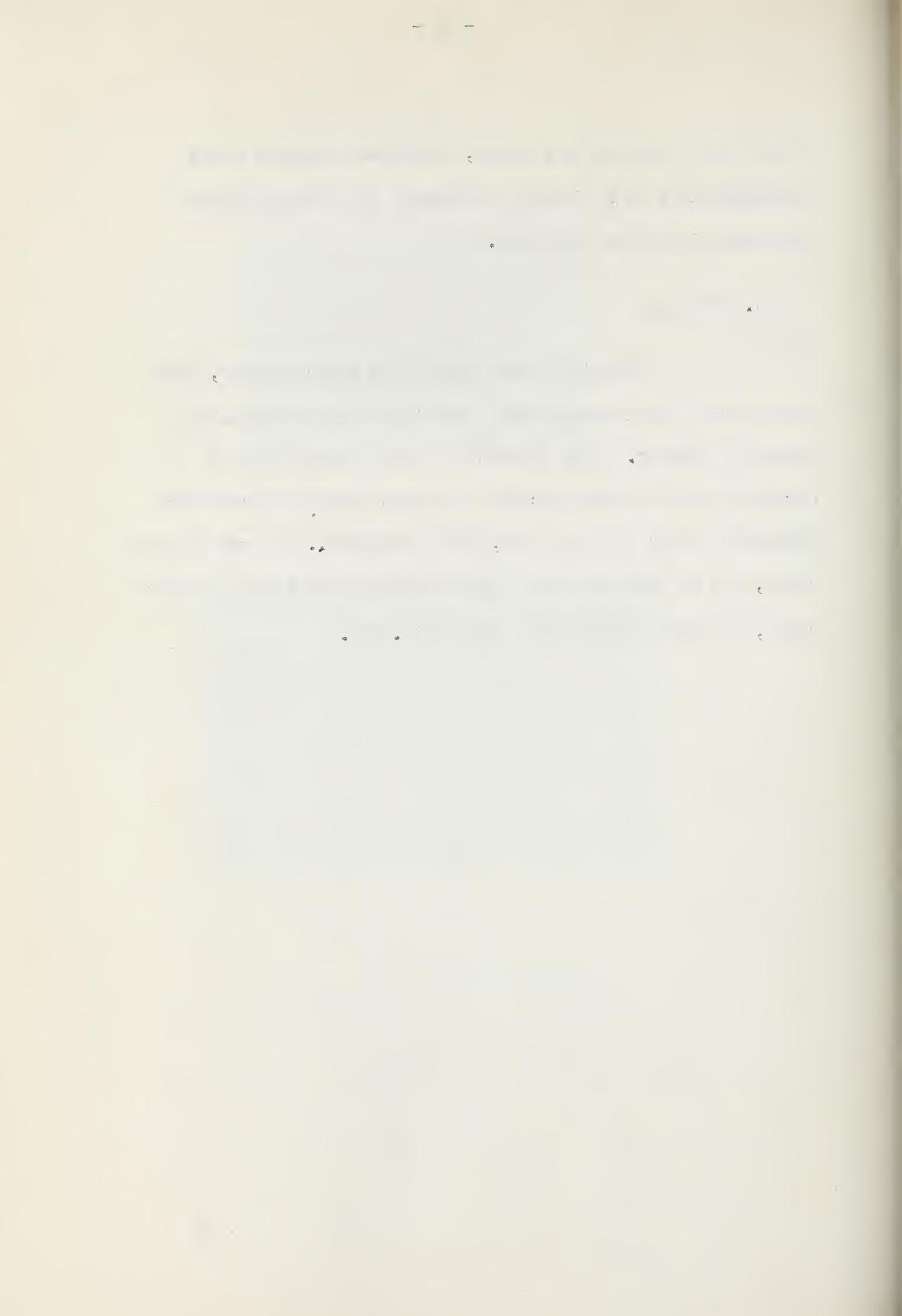
XI. CLUMPING IN CULTURES

One of the most predominant hazards to successful
marrow cultures in fluid media is the occurrence of clumps which,
in the preparation, remove free cells from suspension thereby
drastically reducing the cell concentration, and in the incubating
cultures, make the estimation of population by sampling extremely
inaccurate and increase the objective error in cell counting by
upsetting the even distribution of cells in the hemocytometer.

A. Clumps in the Preparation

Many cells are removed from the original suspension
of marrow by fatty clumps. Stained smears of fat which had risen
to the top of the suspension show that both nucleated and non-nuc-
leated cells are carried along.

Fibrous material from the marrow, reticular tissue
and debris which settles to the bottom of the suspension also
carries with it innumerable cells. A clump of this fibrous material
was recovered, and when fixed, sectioned and stained, showed an
almost solid conglomeration of cells trapped in a febrous network.
The loss of cells through these two channels can be considerable.
In the initial investigations into the technique of suspending
marrow in pure serum, an initial nucleated cell concentration of
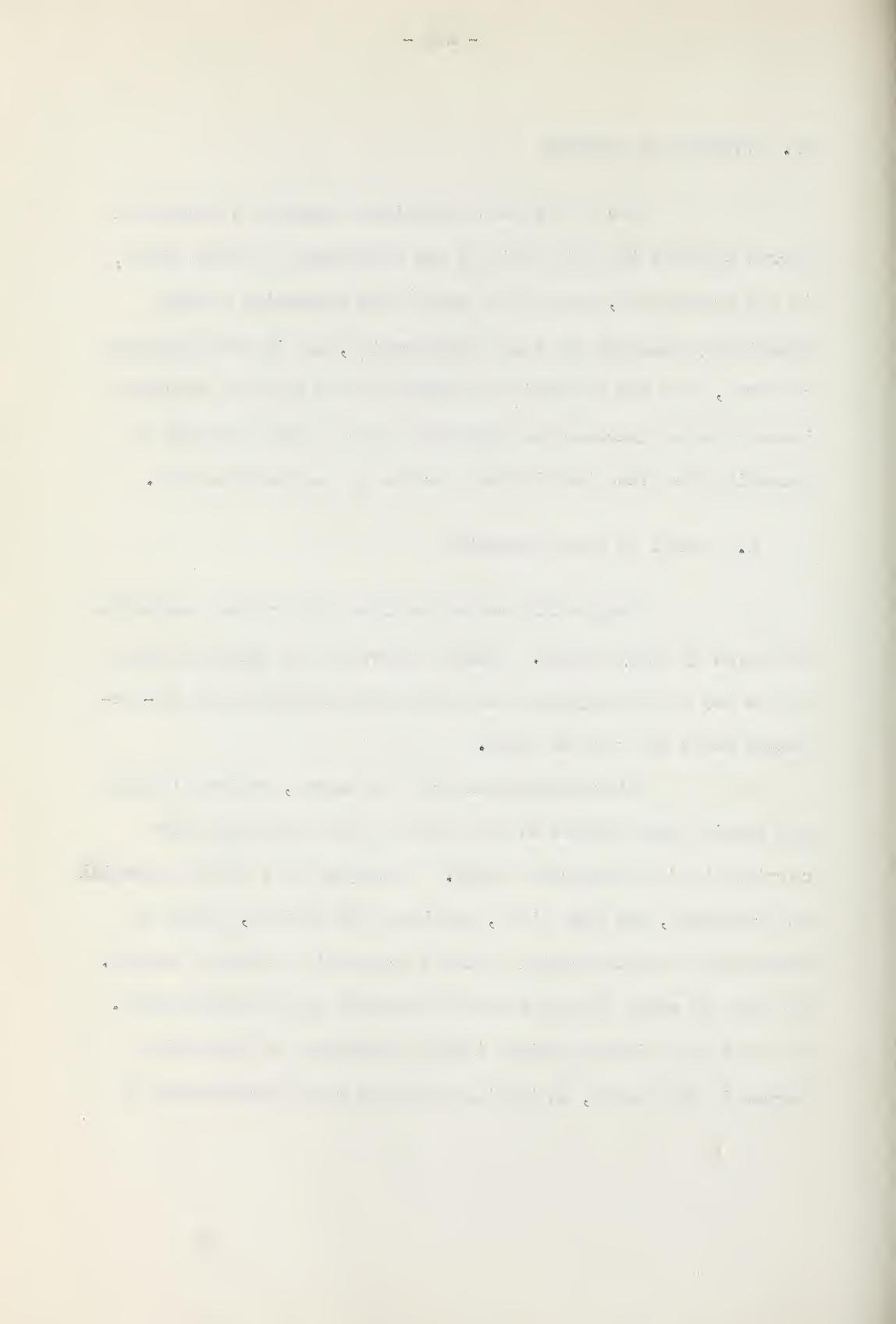
40,000/cu.mm. was decimated to 4,000/cu.mm. by the removal of
fat under very slow speed centrifuging. The large clumps of fat
rising to the surface evidently "filtered" the suspension free
of cells. In a subsequent investigation, an initial nucleated
cell concentration of 41,600 was reduced to 13,500 by fat which
was removed under centrifical force developed at 1,500 r.p.m.
The reduction of the fat content of marrow tissue by hemorrhage
and the removal of fat from the suspension by centrifuging before
large droplets can form from smaller ones minimizes the loss of
cells.

The suspending of marrow cells in pure serum often
results in the loss of the majority of nucleated cells by the
sudden appearance of fibrin. The addition of a bloody tissue rich
in fibrinogen to serum rich in thrombin results in a formation of
fibrin which removes many cells from suspension. In two cases,
nucleated cell concentrations of the magnitude of 50,000/cu.mm.
were reduced to 5,000/cu.mm. The addition of heparin to the
initial suspending serum prevented the formation of fibrin, and
the loss of nucleates was reduced from 90% to 30%.

B. Clumping during Incubation

The appearance of clumps during incubation is a
disturbing factor to the success of the culture because it tends
to destroy the accuracy of the cell counts. They were first
noticed in cultures prepared from hypoplastic marrow tissue when
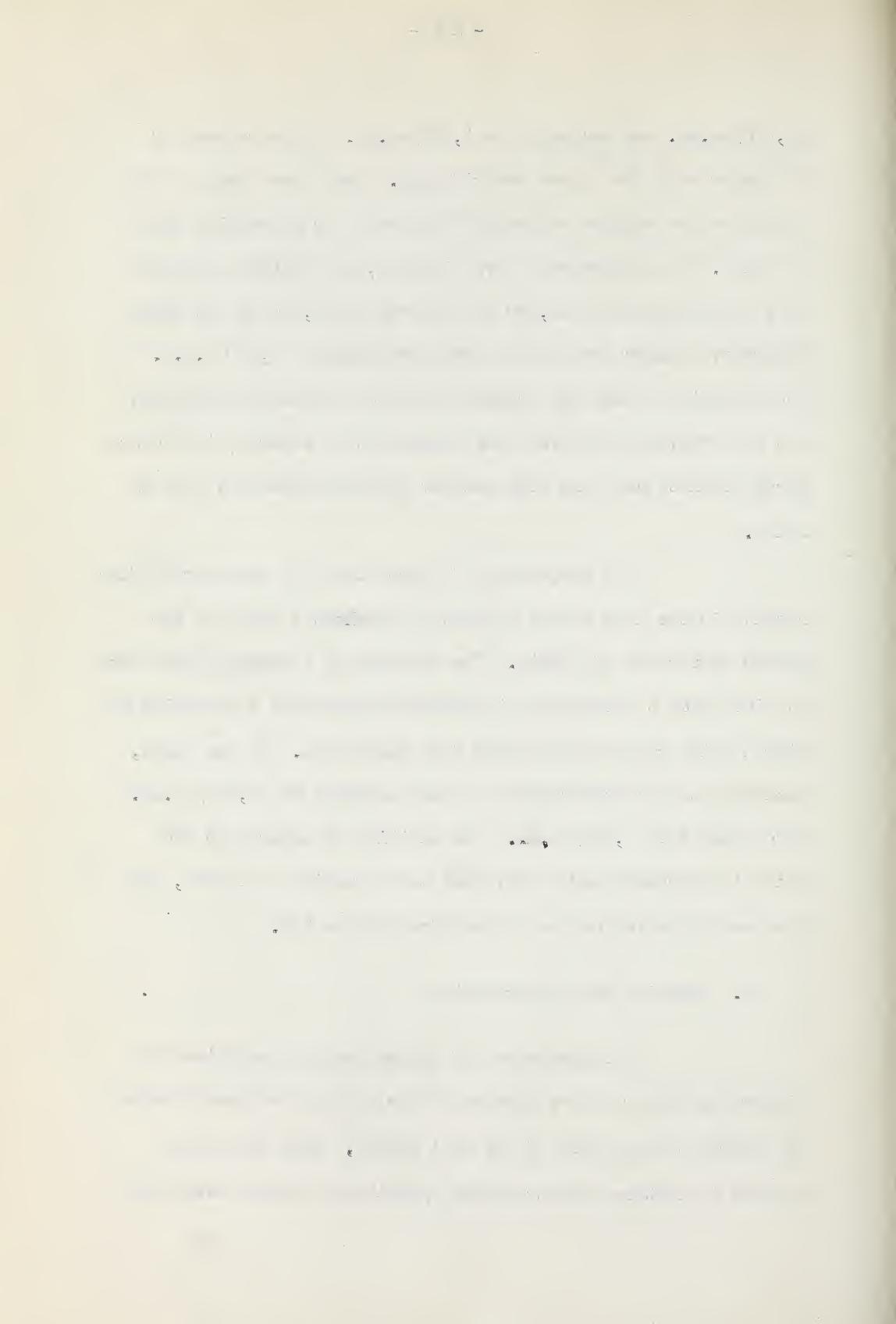
Figure 30. The Trapping of Hematopoietic Cells by

Fibrogenic Activity.

Photomicrographs of smears stained with
May-Grünwald Geimsa stain show what appears to be
marrow cells trapped in a tangle of fibers which
seemingly take origin from "fibroblastoid" cells.

Figure 92. The Recovery of Tetramethyl Oxide by Solvent Activity.

Recrystallization of tetra oxides mix-
distributed values while gold and appears to be
charge while trapped in a sample of fiber which
homologous are stable from "crystallized" cells.
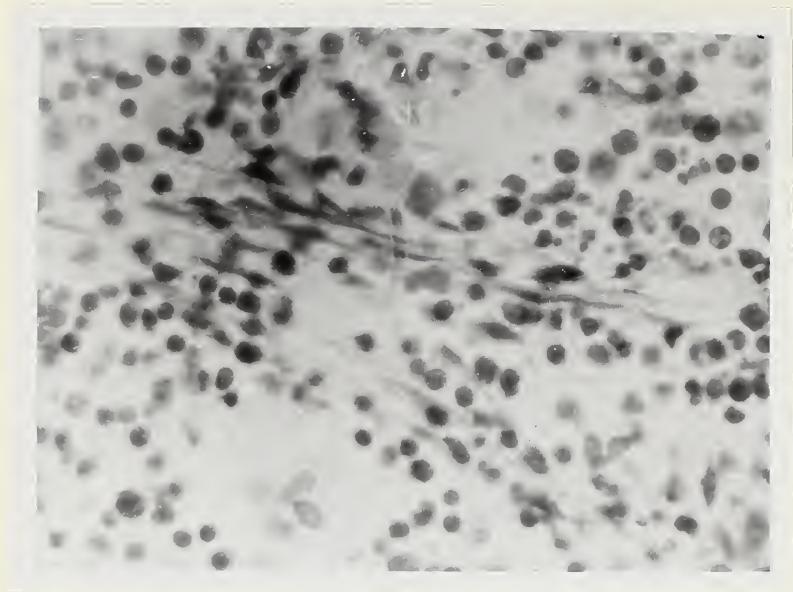
Figure 31. Fibrogenic Activity in Clumps.

           Photomicrograph of a section
(stained with Hematoxylin and Azur II Eosin)
of a clump recovered from an unagitated
culture. Many marrow cells and cells with
spindle-shaped nuclei and long cytoplasmic
processes are evident.

Figure 19. Terminal moraine in Canton.

Photomicrograph of a section (crossed nicols) illustrating the characteristic appearance of an undisturbed section, with matrix among the rock fragments essentially undisturbed.
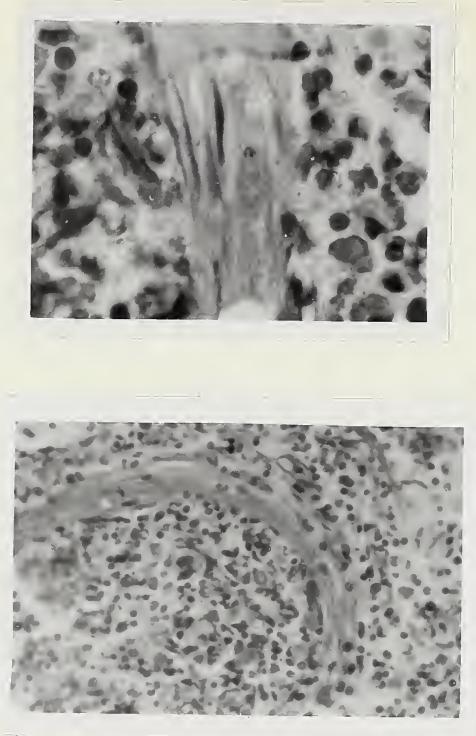
Figure 32. Organized Tissue.

Photomicrograph of a
section (stained with Hematoxylin
and Azur II Eosin) from a clump
recovered from an unagitated
culture. Many marrow cells are
evident. Running through the clump
is what appears to be organized
tissue of parallel fibres and many
spindle-shaped nuclei.

Figure 60. Detention Houses.

The photograph of a typical detention unit house of the residence type is shown. A series of these units are constructed. They are kept well maintained, showing somewhat the class of home provided in a detention house of the sort. There are many small frame houses of this type.

firm, small white bits of tissue formed which could not be completely resuspended by agitation. Histological studies revealed that the clumps were largely cellular in composition but the framework or connecting elements could not be defined.

On several occasions, in suspensions of marrow cells in serum, loose, small grey clumps of material appeared which resisted resuspension by agitation. However, in those cases where heparin had been added, no gross clumping occurred. Microscopically, however, the picture was reversed. Samples from those cultures which had received heparin were full of clumps of three to ten cells and much fibrous material while samples from the heparin free cultures contained only free cells. Fig. 30 shows many cells held together by fibrous strands. There are indications that the strands are of cellular origin rather than fibrinogenic.

During both incubation without agitation and storage of cell suspensions under refrigerated conditions, the formed elements settle to the bottom of the flask. Gentle agitation affects the resuspension of the cells, but almost inevitably there seems to be a "gathering" of thin strands from the bottom of the flask into a small clump which firmly resists all efforts towards resuspension. Fixing and staining facilitated the observation of many nucleated and non-nucleated blood cells bound together by what appears to be organized tissue. Fig. 31 shows a group of blood cells with many interspersed spindle-shaped nuclei and connecting fibrous strands. Fig. 32 shows a large sheet or cord of apparently well organized dense tissue.

Table 2. The Effect of Acute Hemorrhage on Femoral Bone Marrow of the Rabbit.
(All cultures were prepared following the same procedure.)

Culture	Total	Hemorrhage Nucleates	Reds	Culture	Total	No Hemorrhage Nucleates	Reds
59 A	2800	1350	1450	57 A	4250	475	3775
B	2880	1410	1470	B	4400	485	3915
C	1530	800	730	C	1650	263	1388
D	1580	1080	500	D	1500	288	1213
60 A	1850	1075	775	58 A	2850	600	2250
B	1475	700	775	B	1325	225	1100
C	1350	500	850	C	2800	675	2125
D	1900	750	1150	D	1075	288	788
63 A	9525	6425	3100				
B	9600	5525	4075				
C	6825	3625	3200				
D	6650	3160	3490				
Average 3997		2000	1797	Average 2481		412	2069

Total Cell Conc. = 3997/cu.mm.

Of the total cell population the nucleated cells comprise 55%.

Total Cell Conc. = 2481/cu.mm.

Of the total cell population the nucleated cells comprise 17%.

XII. THE EFFECT OF HEMORRHAGING ANIMALS BEFORE BONE MARROW CULTURES

Cultures prepared from twelve marrows during the autumn of the year proved to be of no value because of the unsuitability of the tissue. Large amounts of fat and debris were encountered, and the low concentration of nucleated cells in suspension made the suspension unusable for culture purposes. The previous researcher in this laboratory (Nelson) recalled similar experiences during the autumn of the previous year when the project was newly instituted.

In an attempt to replace the labile marrow fat with hematopoietic tissue, the rabbits were hemorrhaged by cardiac puncture. Using the figures obtained in the initial cell counts of twenty cultures from five marrows, three from hemorrhaged and two from unhemorrhaged rabbits, where the cultures had been prepared by an identical method, the average concentration of cells in suspension was almost doubled after hemorrhage and the nucleated cells which had previously comprised 17% of the total were now 55% of the total population.

The preparatory procedure was eased considerably with the absence of large amounts of fat and fibrous material. The marrow before aspiration grossly appeared red, bloody and friable where previously it had seemed to be made up of greasy, yellow masses.
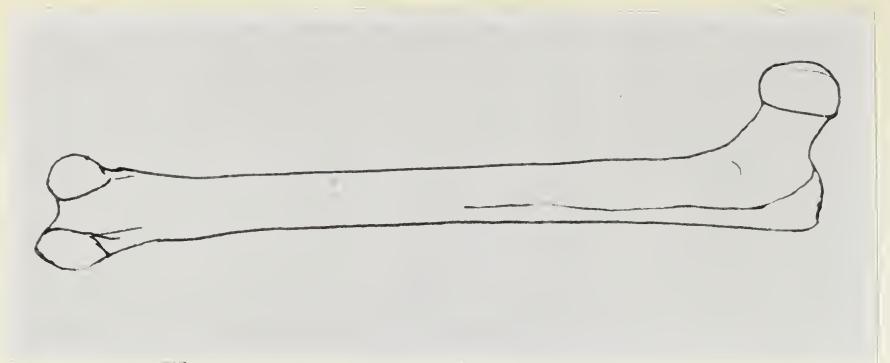
Unhemorrhaged
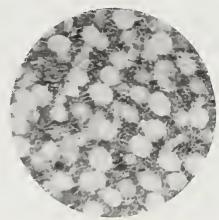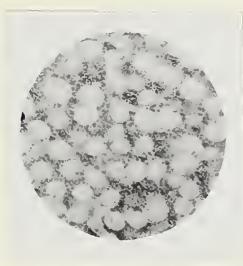
Hemorrhaged
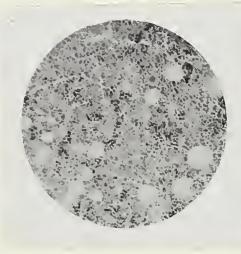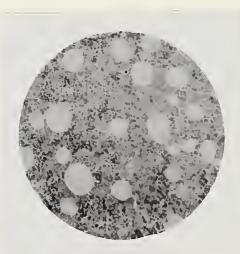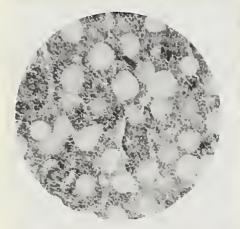
Figure 33. The Effect of Hemorrhage on Rabbit Bone Marrow.

Figure 2. The effect of overstorage on Baltic bone marrow.

To provide direct evidence of the change in bone marrow stimulated by acute hemorrhage, several whole femurs were fixed in Maximow-Zenker solution, decalcified in 5% formic acid, embedded, sectioned longitudinally and stained with Hematoxylin and Azur II Eosin. Fig. 33 compares hemorrhaged marrow with small lipoid islands and fairly large areas of cellular tissue, to unhemorrhaged marrow with large fat deposits and only thin strands of cellular tissue. It is also of interest to note that the femurs of unhemorrhaged rabbits show the greater concentration of cellular material in the head and distal end of the cavity while the shaft marrow is relatively hypoplastic, and in the case of hemorrhaged rabbits, the proximal marrow is relatively more cellular than the distal marrow.

XIII.   SOME STUDIES OF POSSIBLE COUNT ERRORS

The method of sampling and diluting at the same
time from all cultures being incubated during that time, aroused
some speculation as to whether there was any hematopoietic
activity or cell breakdown occurring in the pipettes which awaited
counting at room temperature. Since two samples were taken from
each culture within a few moments and since one sample from each
culture, comprising one "set," was counted before the next "set"
was begun (i.e., A, B, C, D -- D, C, B, A), a comparison could
be made of the cell concentrations in each set of pipettes. The
figures for the initial and final counts were averaged in Table 3.
It can be seen that the average number of cells counted from the
pipettes of the first set was almost identical, in both initial
and final counts, to the average number from the pipettes of the
second set.

Proof that maturation was not occurring in the
pipettes is shown in Table 3 where the ratios of nucleated and
non-nucleated cells are given. The ratio, in both initial and
final counts, is the same in both the first and second set.

Since any one pipette, after shaking in the
mechanical shaker, is used to charge one side of the hemocytometer,
and after further shaking is used to charge the other side, it was
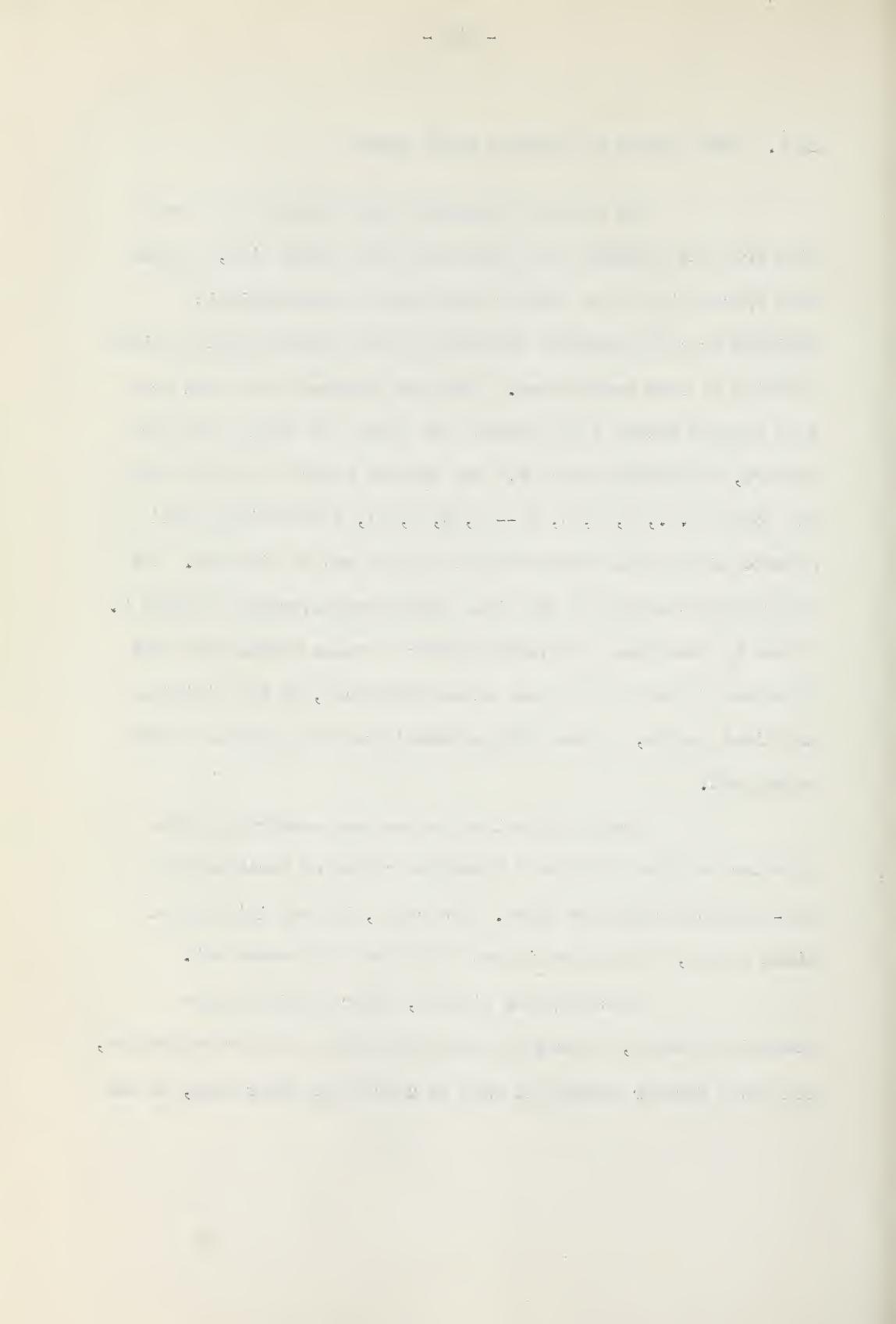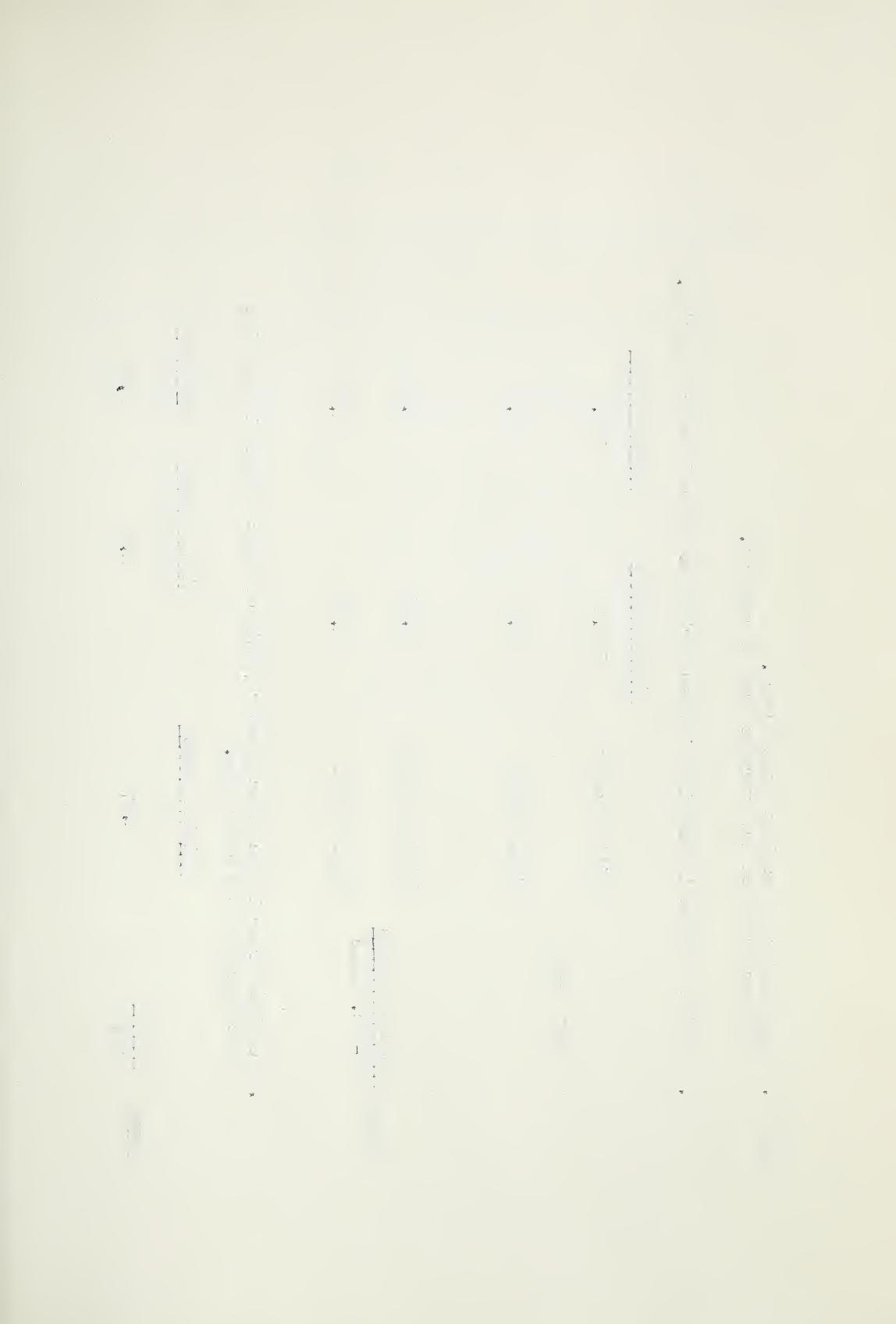
Table 3. Some Studies of Possible Count Errors.
(Figures presented are averages from 12 cultures.)

A. Possible Changes in Sampling Pipettes during the Interval before Counting.

		Pipette Set 1	Pipette Set 2
Total Counts	Initial Counts	144.2	148.0
	Final Counts	98.8	96.4
RATIOS Nucleated Cells / Non-Nuc. Cells	Initial Counts	3.45	3.48
	Final Counts	1.29	1.49

B. Possible Error in the Hemocytometer (Ratio of Cells counted in one side as compared to the other side.)

	Initial Counts	Final Counts	Combined
RATIOS Side A / Side B	1.07	1.00	1.04

felt that comparing the number of cells counted in each side
of the hemocytometer would indicate not only possible differences
in the chambers themselves, but the traumatic effect of the
pipette shaker on the cells, and the effect of the five minute
delay between the charging of the chamber and counting as compared
to no delay. Table 3 shows that for both initial and final counts
the difference is slight.

XIV. THE EFFECT OF CANCEROUS SERUM

Serum from two cancer sufferers who showed the
"anemia of cancer" and normal human serum from dental and
medical students were similarly heated to 56.6° C. for one
hour, and then exposed to repeated aliquotes of washed
rabbit erythrocytes.

Eight cultures were prepared using the marrows
of two rabbits, four cultures were suspended in normal serum
and four in pathological serum. However, residual agglutinins
in the cancerous serum all but destroyed the cultures. The
erythrocytes were observed to be in groups of two and three with
free cells rare. Nucleated cells were in large clumps which
made counting next to impossible. Therefore no results are
presented.

DISCUSSION

I. CULTURES UNDER VARIOUS CONDITIONS

The greatest production of erythrocytes was recorded for cultures in a medium of two parts balanced salt solution and one part native serum, the cultures having been prepared atraumatically. It is interesting to note that marrow incubated under identical conditions in the same medium, but prepared by a process consisting of repeated high speed centrifugings and other traumatic manoeuvers was much less active erythropoietically. This is probably due to damage inflicted on the erythroid precursors by the high centrifugal force and packing. However the survival of nucleated cells indicates that the loss of cells in the traumatic preparation is less marked than under the atraumatic method. It is possible that the

packing of cells damaged the marrow cells enough to upset the
metabolism without actually destroying them.

The erythropoiesis in the cultures in mixed
balanced salt solution and serum medium prepared atraumatically
was far superior to red cell production in pure native serum,
heated native serum, native plasma and human serum, all of which
had been prepared and cultured in a similar atraumatic manner.
The first impression, that balanced salt solution and serum is
superior to pure serum or plasma, the normal liquor of the body,
does not seem logical. Nevertheless, considerable attention was
given to the subject of growth inhibitors in adult serum by Carrel
and his co-workers (13a) and later by Gey and Gey (31). They
found that placental serum and plasma supported fibroblast growth
while adult blood fluids did not. It is not improbable that the
low level of erythropoietic activity found in cultures of native
serum is due to the presence of this depressent principle. The
answer to the comparatively poor proliferative activity may lie in
another direction. The serum is extremely important in preserving
the proper pH of the body. One of the buffer-pairs is the sodium
bicarbonate-carbonic acid relationship. To maintain a physiological
pH by means of this buffer, it is necessary that the gas lying over
the serum should have a carbon dioxide content identical to the
content of the erythropoietic tissue. Since the circulation of the
marrow is somewhat sluggish and has even been termed stagnant, the

carbon dioxide tension is undoubtedly considerably higher than is reflected in the general venous circulation and the pH of the environment of erythropoietic activity is lower than the classical 7.4 of mammalian life. In the cultures with pure serum, no attempt was made to modify the composition of overlaying gases and as a result, carbon dioxide undoubtedly diffused from the serum, the buffer equilibrium shifted, the pH of the culture medium rose and cell proliferation ceased (13). No specific studies were made on the subject, however, and no data is available to support this reasoning.

The composition of the balanced salt solution was such that in equilibrium with atmospheric air, a pH within physiological limits was maintained, and no interference of cell proliferation occurred due to acid-base upset.

Serum has the advantage of eliminating possible contamination by impure chemicals or non-sterile medium, and at the same time, makes possible the simplified processing of the marrow in the preparation of the cultures. To successfully employ serum as a culture medium, some provision would have to be made for the volumetrically accurate mixing of gases and their equilibration with the culture medium.

Cairns and Lajtha (7) made extensive studies on the loss of nucleated cells "in-vitro" and found that the major

leak was due to sticking to glass surfaces. The sticking required
a heat labile factor which could be destroyed by maintaining the
serum at 56° for one hour. No difference was recorded in erythro-
poietic activity between cultures in native serum medium and heated
native serum medium. However, the nucleated cells survived in the
heated serum to the extent of 80% while in unheated serum only 55%
of the nucleates survived.

Plasma supported erythropoiesis to a greater degree
than serum and the survival of cells was of a higher order. This
seems to indicate that some factor present in plasma and lacking in
serum contributes both to production of non-nucleated cells and
proliferation of nucleated cells.

Normal human serum as a culture medium appears to
be unacceptable in that erythropoietic activity was almost non-
existent and that over half of the nucleated cell population
disappeared in the four hour culture period. This may be due to
the presence in the "foreign" serum of some components which are
distinctly toxic to rabbit cells and affect their breakdown. In
fact, non-specific agglutinins and hemolysis prohibit the use of
this type of medium before complex heating and fixing treatments
have been carried out. Heating of the serum to 56° C. prevents
hemolysis and reduces the degree of agglutination. Repeated
treatments with aliquotes of washed rabbit peripheral blood cells
affects the fixation and removal of agglutinins. However, even

the use of excess red blood cells does not assure the complete
removal of all agglutinins; residual traces cause the clumping
and loss of both erythrocytes and nucleated cells.

II. THE EFFECT OF HEPARIN

The addition of heparin to cultures in native
serum medium decreased the survival of nucleated cells and
lowered the erythropoietic activity. The addition of heparin
to cultures prepared with normal human serum medium has exactly
the opposite effect. The loss of nucleated cells is decreased
from sixty percent (in the controls) to a mere ten percent and
erythropoietic activity is almost doubled. The reason for this
is difficult to determine. Heparin is a normal tissue constituent
and aside from its anti-coagulent effect, is free of toxic actions.

The apparent depressent effect of heparin in
native serum cannot be attached to the small amount of phenol
preservative or impurities in the heparin since these would
similarly disturb cells cultured in human serum. The explanation
may be the fact that human serum from normal young men is a more
favourable medium for growth than rabbit serum. However, human
serum, even after heating, contains agglutinins which destroy
cells and mask or prevent any proliferation. The heparin, even
if it had a slight depressent effect directly on the cells (which
would account for the loss of nucleated cells and lower erythropoiesis

in native serum medium) might counteract the agglutinins thus
allowing the benefits of the superior medium to be demonstrated
by the increased production of erythrocytes and the minimizing of
nucleated cell losses.

III. THE EFFECT OF AGITATING CULTURES

Slight continuous agitation during the incubation
of cultures resulted in a very slightly greater increase in erythro-
cytes than occurred in unagitated cultures. However, the survival
of nucleated cells in agitated cultures was notably higher. The
major reason for the lower nucleated cell concentration in unagitated
cultures is the formation during incubation of large clumps of
material in which many nucleated cells appear to be trapped. The
question of a need for a growing surface or framework for cells "in-
vitro" is controversial. Carrel (14) insisted that without a
framework, the cells could not multiply. Fell (28) stated that
cells in her experience are like cliff dwellers and require something
to lean against. On the other hand, Osgood (56) and Plum (65) and
others found that marrow cells can be cultured in suspension. It is
possible that in agitated cultures fibroblast-like elements are
inactive because of the absence of a growing surface. However, in
unagitated cultures, the cells settle to the bottom of the flask,
the fibroid cells become active and the resultant clumps trap
nucleated marrow cells.

IV. THE USE OF LIVER EXTRACT

The number of workers who have employed or studied liver extracts in cultures of blood forming tissues is almost infinite. Notably, Plum (66) found that the use of liver extract considerably increases the production of erythrocytes. However, Nelson (43) in this laboratory was unable to show any advantage in using the available preparations.

In two series of cultures, using different amounts of extract, erythropoietic activity was lowered below the level of activity in the controls. In one series the rate of nucleated cell proliferation was lower than in the control. In the other series (less dependable since only two cultures were included) the nucleated cell concentration remained higher where liver extract had been added. There is little that can be said to explain this lack of agreement with earlier workers, except that experimental conditions differed enough to produce the inconsistency.

V. THE EFFECT OF EMBRYO EXTRACT

The widespread use of tissue extracts in cultures of cells "in-vitro" stems from the work of Carrel (9) who first employed embryo extract in the study of connective tissue.

The cell free juice from an eight day chick embryo was employed in cultures of rabbit bone marrow in native serum. Contrary to the control cultures which showed increases in both nucleated and non-nucleated cell concentrations, the nucleated cell concentration in the cultures which had received embryo extract dropped precipitously and the erythrocyte population decreased considerably.

The large destruction of cells is due undoubtedly to the addition of a foreign protein, that is, material from another species. Carrel in his original article stated that the activity of tissue extract is specific for that species. Not only was there a lack of stimulation of the rabbit cells by the chick material but probably a definite destructive action.

VI. THE EFFECT OF COLCHICINE

The alkaloid, colchicine, has the property of inhibiting mitotic division. This substance was employed to facilitate the study of the manner of erythrogenesis. A solution of colchicine was added to culture so as to provide a final concentration of 1/125,000 as suggested by previous workers (68). As was suspected, nucleated cell proliferation was almost completely halted, while the erythrocyte concentration increased, the rise being only slightly less than in the control. These results

completely support the results obtained by Plum (68) in a similar
experiment.

Plum logically theorized that if erythrocytes are
produced even when mitotic activity is at a standstill, then this
production must be completely unrelated to mitosis. This is one
of the strong arguments in favour of budding and cytoplasmic
constriction as the mode of normoblast-erythrocyte maturation.

VII. THE EFFECT OF FAT REMOVAL

The evident increase in erythropoietic activity in
cultures free of fat can be explained by the fact that lipids seem
to have an inhibiting effect on proliferating cells. The removal
of the fat allowed erythropoietic activity to proceed relatively
unimpeded.

VIII. THE USE OF PHYTOHEMAGGLUTININ

The use of bean extract containing a strong non-specific
agglutinin to reduce the initial concentration of red cells in culture
was initiated largely as a result of Osgood's statement (54) that
red blood cells in culture tend to inhibit successful growth.

The results of two series indicated that it was
valuable for two reasons. By reducing the initial erythrocyte count
to a very low number, subsequent growth was not masked by a relatively
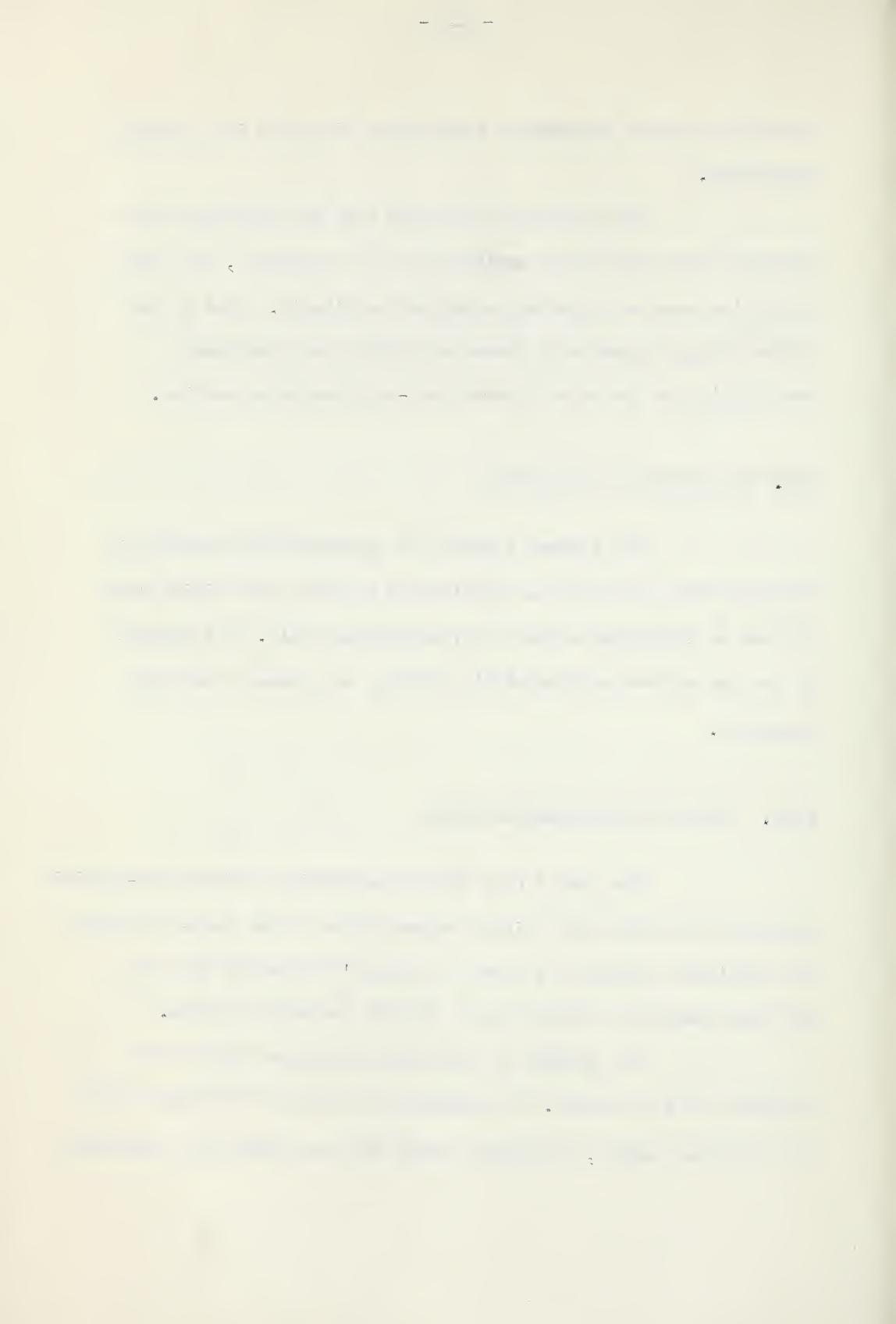
large concentration of inert cells. Secondly, due either to
direct stimulation or to the removal of a large number of erythro-
cytes, the proliferation of cells in culture was intense. However,
later work indicated that the supposed growth was falacious, an
artifact dependent on the method of cell counting. Following the
processing of the marrow with bean extract, many small groups of
two or three erythrocytes remained in suspension. Some of these
were drawn into the sampling pipettes for the initial cell counts,
and under the trauma of the pipette shaker, hemolyzed, were not
seen in the hemocytometer and the result was a false low count.
During the incubation period the anti-agglutinating power of the
serum medium (73) was exerted and the cell groups dispersed. In
the final counts, the cells which previously had been in groups,
now unattached and less sensitive to trauma, were counted and
interpreted as new cells which had been produced during the
incubation period.

    The bean extract was abandoned as too difficult
and too erratic to apply to this work. The complexities of producing
and maintaining a sterile, active preparation, and applying it in
exactly the necessary amount would require separate extensive
investigations which were not practical at the time.

IX. THE STORAGE OF CELLS BY REFRIGERATION

A. The Effect of Storage at 4° C.

During the first 24 hours of refrigerated storage the concentration of nucleated cells dropped considerably. In the next 24 hours, there was only a slight drop. The initial drop can probably be attributed to the breakdown of cells which had been damaged in the preparation of the cultures. Once these cells had disappeared there was little change in the nucleated cell population.

Of two series of cultures studied, the erythrocyte concentrations increased in both 24 hour periods in one series and dropped in the first 24 hours in the second series. (The second series was not carried to 48 hours.) It is difficult to suggest any possible explanation for the apparent production of erythrocytes at a temperature of 4° C. It is hardly possible that metabolic processes could proceed under such contrary conditions. In an effort to advance some theory, the possibility of the last step in maturation occurring might be entertained; that is, some cells may be "on the edge and simply waiting to fall off." This line of reasoning might in addition account in part for the disappearance of nucleated cells.

B. The Ability of Cells to Grow after Storage at 4° C.

The nucleated cell concentration of cultures which had not been refrigerated dropped during the four hour culture period. However, in cultures which had been refrigerated, for 24 and 48 hours, the nucleated population increased markedly. This might be explained by the fact that all cells damaged during culture preparation disintegrated during the four hour incubation period of unstored cultures resulting in a decrease in cell concentration.

In cultures which were stored, all the damaged cells had disappeared before the incubation was initiated (and before the initial counts were done) and therefore did not nullify proliferation by undamaged cells during the incubation.

Erythrocyte concentration increased equally in unstored cultures and cultures which had been stored for 24 hours, but decreased in cultures which had been refrigerated for 48 hours.

These studies indicate that under the prevalent conditions, prepared cultures can be stored at least 24 hours without untoward effects. Research into the preservation of blood (72) (for transfusion) suggests that for each temperature of storage, studies are necessary to determine the optimum serum-artificial medium ratio, the ratio of the cells to the fluid component, and the necessity of other additions.

X. ERYTHROPOIESIS BY BUDDING AND CYTOPLASMIC CONSTRICTION

The observation of cells in the culture suspension after incubation which vary in size, shape and form is in complete agreement with the detailed reports of Victor Emmel (23) who made the first (and only) detailed continuous direct study of the maturation of the normoblast to the adult erythrocyte. New red cells are, he observed, generally spherical in shape and only later acquire the characteristic biconcave shape. Variation in the size of cells is not uncommon. Poikylocytes are present as the direct result of the budding process. New erythrocytes have a "tail" which is the remnant of the attachment to the normoblast.

The observation of cells in the counting chambers which appear to be a stage of the budding process was not uncommon. These varied from what could be called an excentric nucleus to a bicellular form in half of which the nucleus remains, and the other half of which could be termed a "neoerythrocyte." At no time was an erythrocyte observed at the point of breaking free from the parent normoblast. The reason for this can be found in the rather traumatic shaking to which the sampling pipettes were subjected, a process which was certain to complete any imminent division.

XI.   CLUMPING IN CULTURES

The appearance of clumps during preparation of cultures and the resultant loss of cells is undoubtedly dependent upon the amount of fat and fibrous tissue present in the marrow. The use of tissue which is highly erythropoietic and relatively free of lipoid material is the obvious way to minimize the undesirable occurrences. The use of pure serum in place of balanced salt solution in the preparation of the cultures facilitates the removal of the fat without undue loss of nucleated cells.

Clumping in cultures can be associated with fibrin formation or fibroblast-like activity. The formation of fibrin in serum is dependent upon the transport of fibrinogen into the medium with the marrow tissue. Strict adherence to the procedure which outlines the primary suspension of the cells in heparinized serum which is later drawn off, presumably with most of the fibrinogen, minimizes the possibility of fibrin formation. The production of fibrous material by cellular activity seems to depend upon the concentration of fibrogenic cells in suspension and the availability of a growing surface. The use of cellular marrow reduces the incidence of clumping, probably because fewer fibrogenic cells are present. The continuous agitation of cultures prevents settling of cells to the bottom, and without growing surfaces, the cells remain inactive.

It is distinctly possible that where faults appear in the siliconed surfaces of the culture flasks, fibrogenic cells become attached, and as a result fibrous material and clumps appear. Similarly, if several nucleated cells should stick together, or enter the culture as a group in which fibrogenic cells are lodged, fibrous material would be produced enlarging the clump and trapping more cells.

CONCLUSIONS

1. The preparation and culture of hematopoietic tissue
"in-vitro" is a complex and difficult procedure which is subject
to many variable influences. The use of balanced salt solution
as a medium does not facilitate easy processing of the marrow
and introduces the possibility of bacterial and chemical contamin-
ation. Native serum provides a valuable means for a simple, rapid
and relatively atraumatic preparation of the tissue for culture
since it permits easier separating of fat from cells without
severe mechanical processing.

The use of normal human serum as a culture medium
for rabbit marrow cells is complicated by the existence of
agglutinins and hemolysins which destroy the culture. These can

be eliminated only by a prolonged and somewhat unreliable procedure.

The assessment of cell proliferation by hemocytometric techniques is long and tedious and might be replaced profitably by a microculture method which would allow direct observations of cells in culture.

2. Pure serum as a culture medium supported erythropoiesis to a lesser degree than serum fortified balanced salt solution, probably due to the loss of carbon dioxide and resultant shift of pH in the former method.

3. In cultures prepared by traumatic methods (prolonged high speed centrifugings), erythropoietic activity was notably less in identical cultures prepared by relatively atraumatic methods.

4. Heparin used as an anti-coagulent has a slight stimulatory action on rabbit marrow cells in normal human serum and a slight depressent effect on rabbit marrow cells in native serum.

5. There is no indication that the liver extract used has any stimulating effect on marrow cells "in-vitro."

6. Under the experimental conditions, storage of prepared marrow cultures in native serum at $4°$ C. is feasible up to 24 hours.

7. Marrow suspensions appear to multiply equally well whether agitated or not during the culture. However, in unagitated cultures,

fibre forming cells become active resulting in clumps of "organized tissue" which enmesh the hematopoietic cells making reliable counting impossible.

8. Observations made support Emmel's theory of erythrogenesis by budding and cytoplasmic constriction.

SUMMARY

1. An extensive review of the previous works in bone marrow culturing was presented including a critical analysis of the methods employed.

2. Several persistent problems in the physiology of hematopoiesis were reviewed.

3. The development of a new technique for the preparation of bone marrow cultures was described.

4. Data was presented concerning the culturing of marrow cell suspensions in various media.

5. Data was presented from cultures to which various substances had been added, and the results were discussed.

6. The feasibility of storing cultures under refrigeration was demonstrated.

7. Evidence was presented which favours the theory of erythrogenesis by budding.

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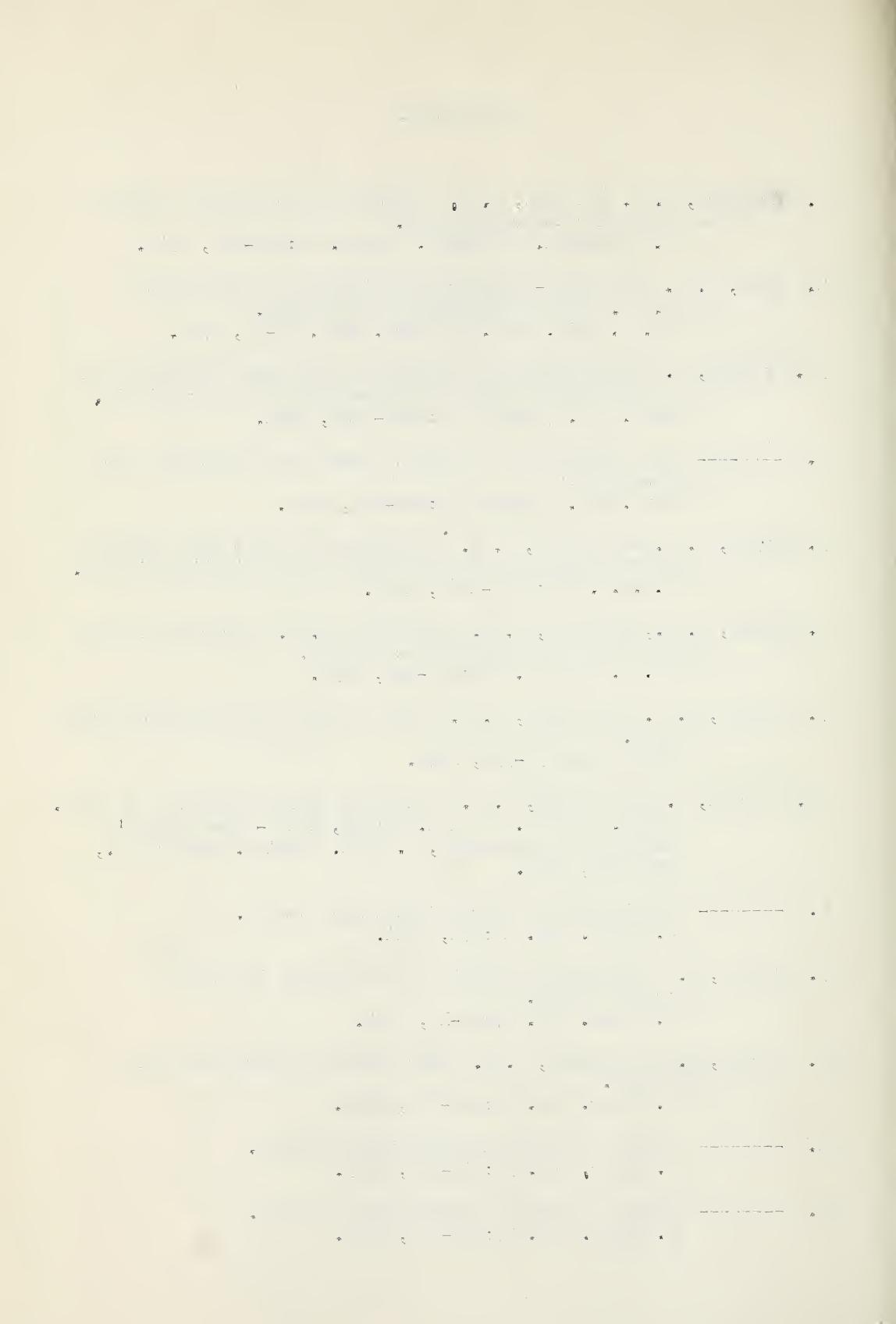
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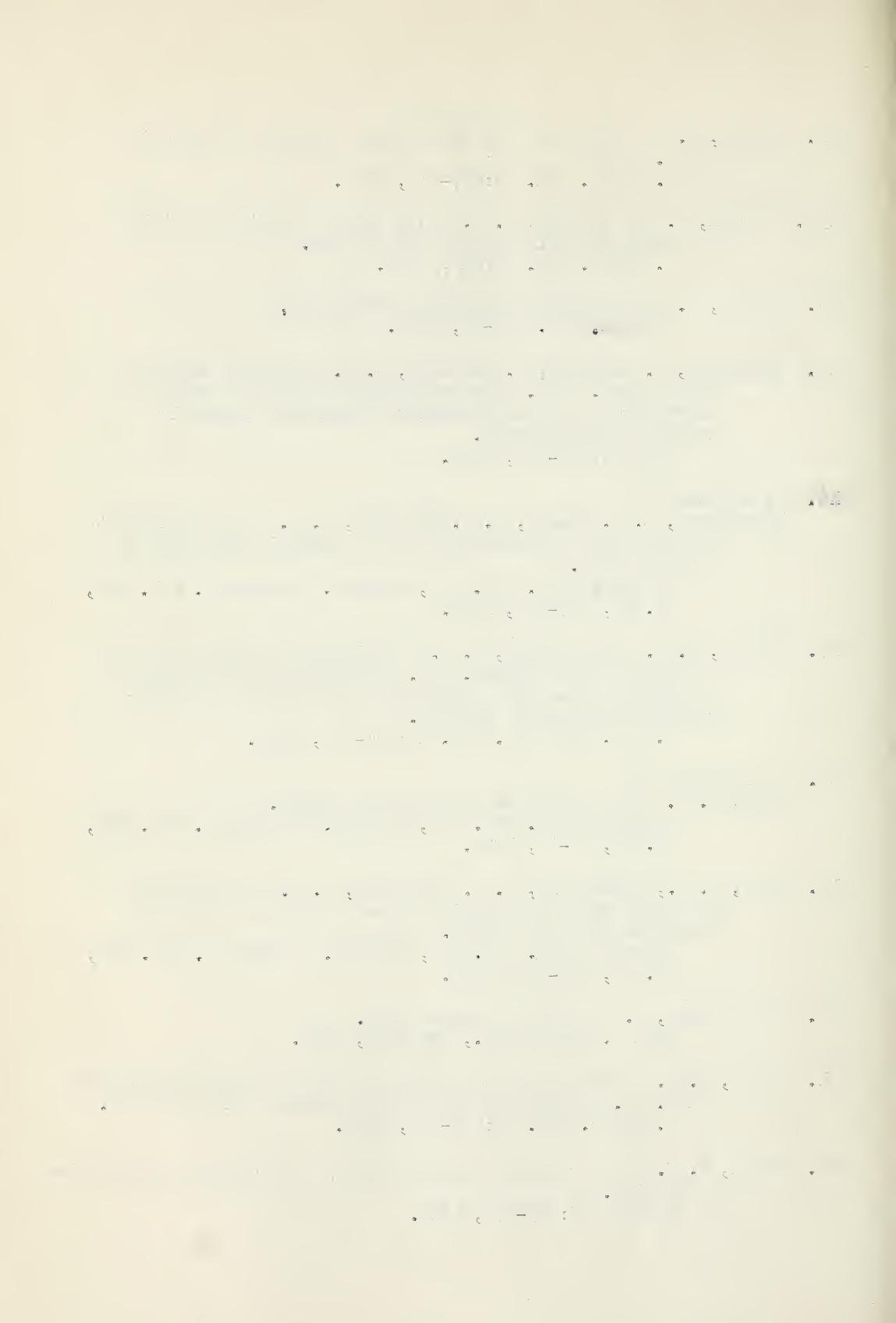
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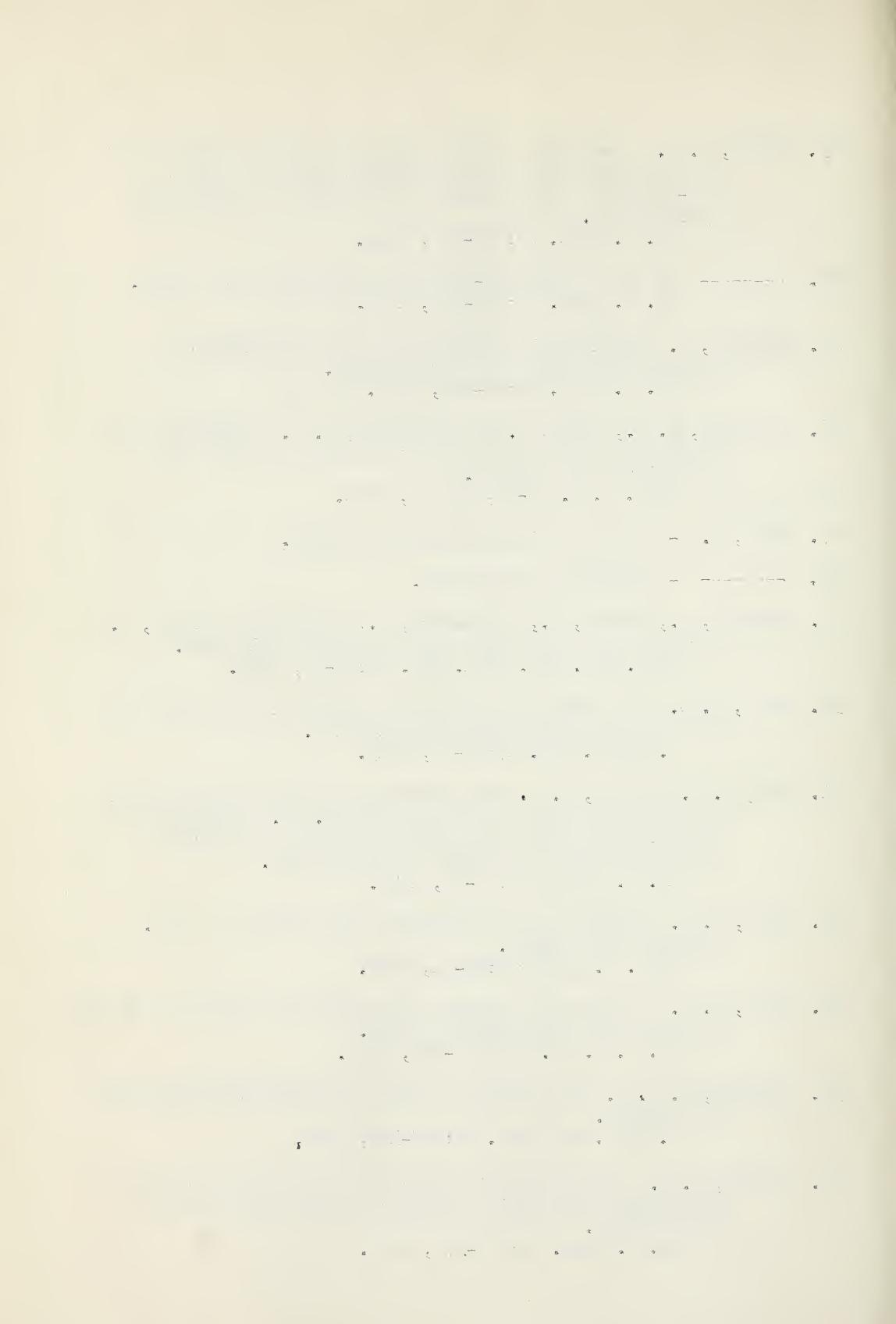
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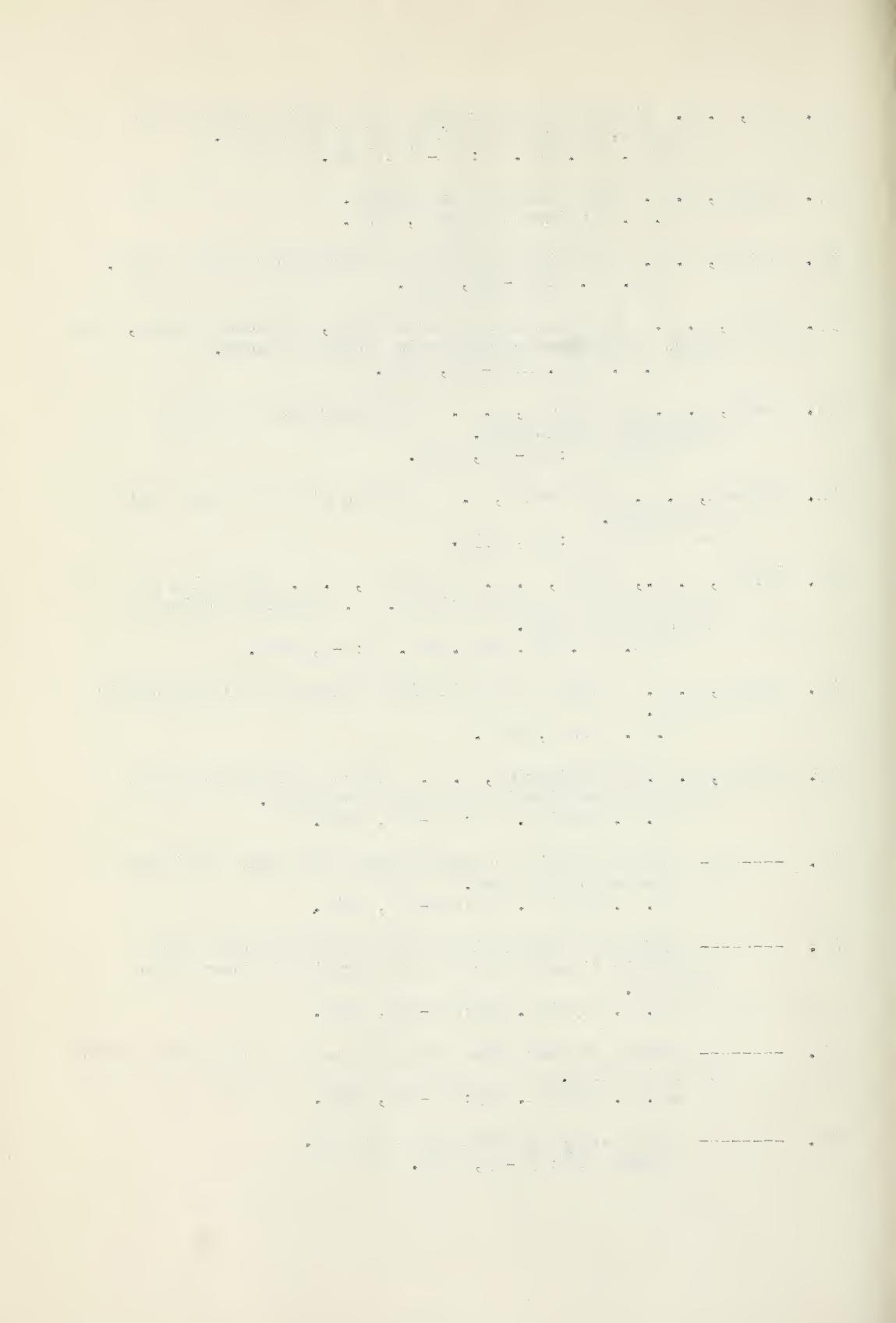
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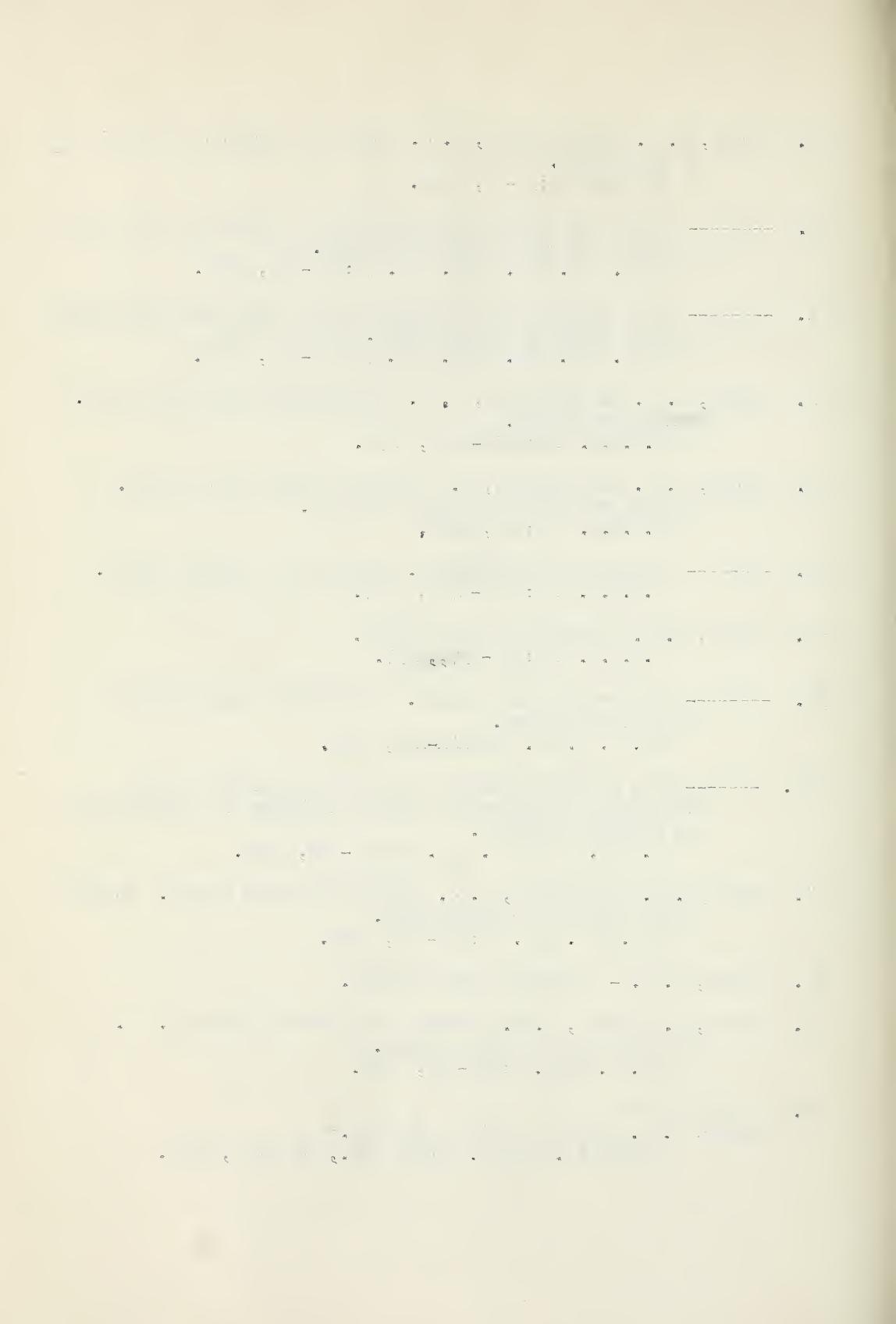
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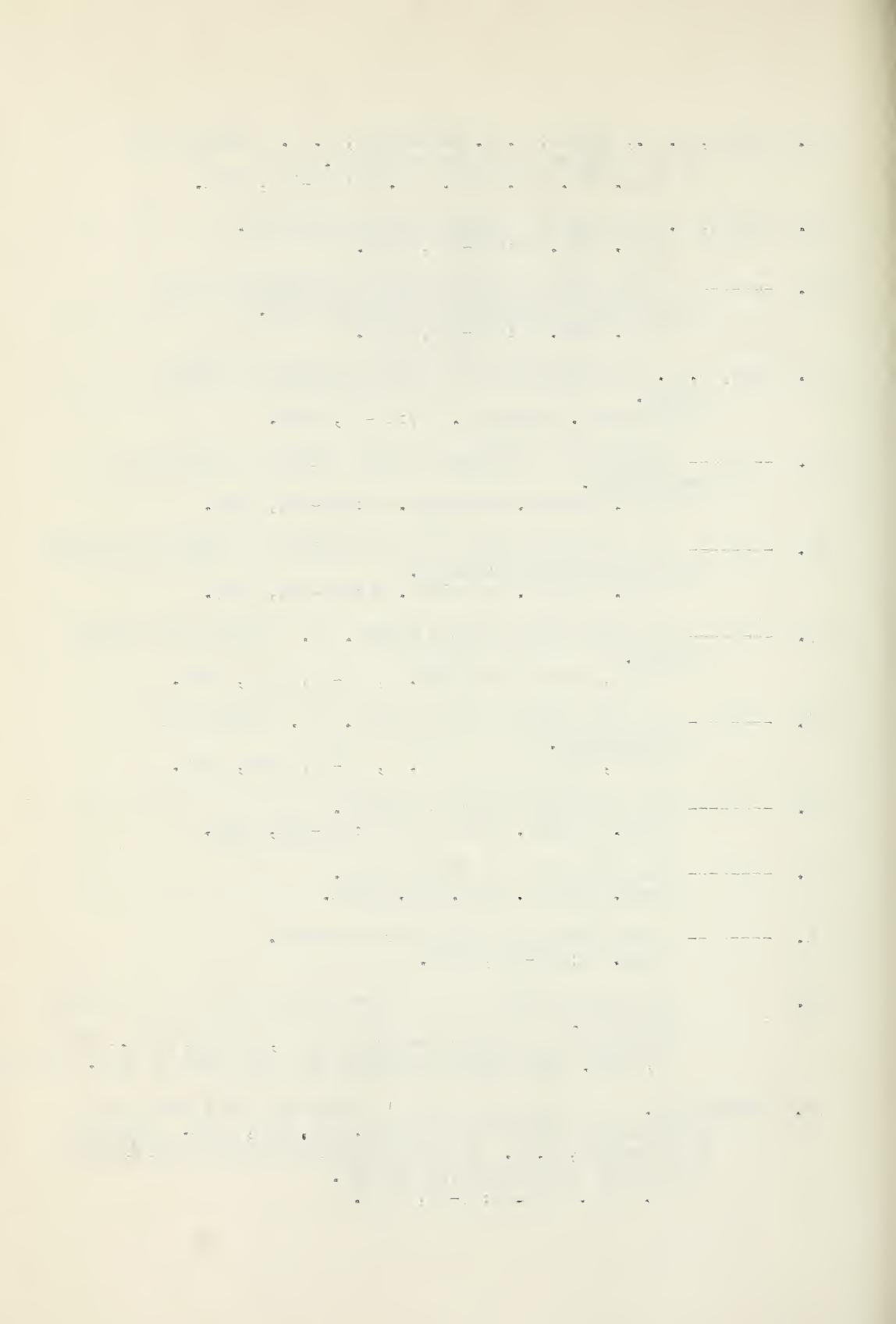
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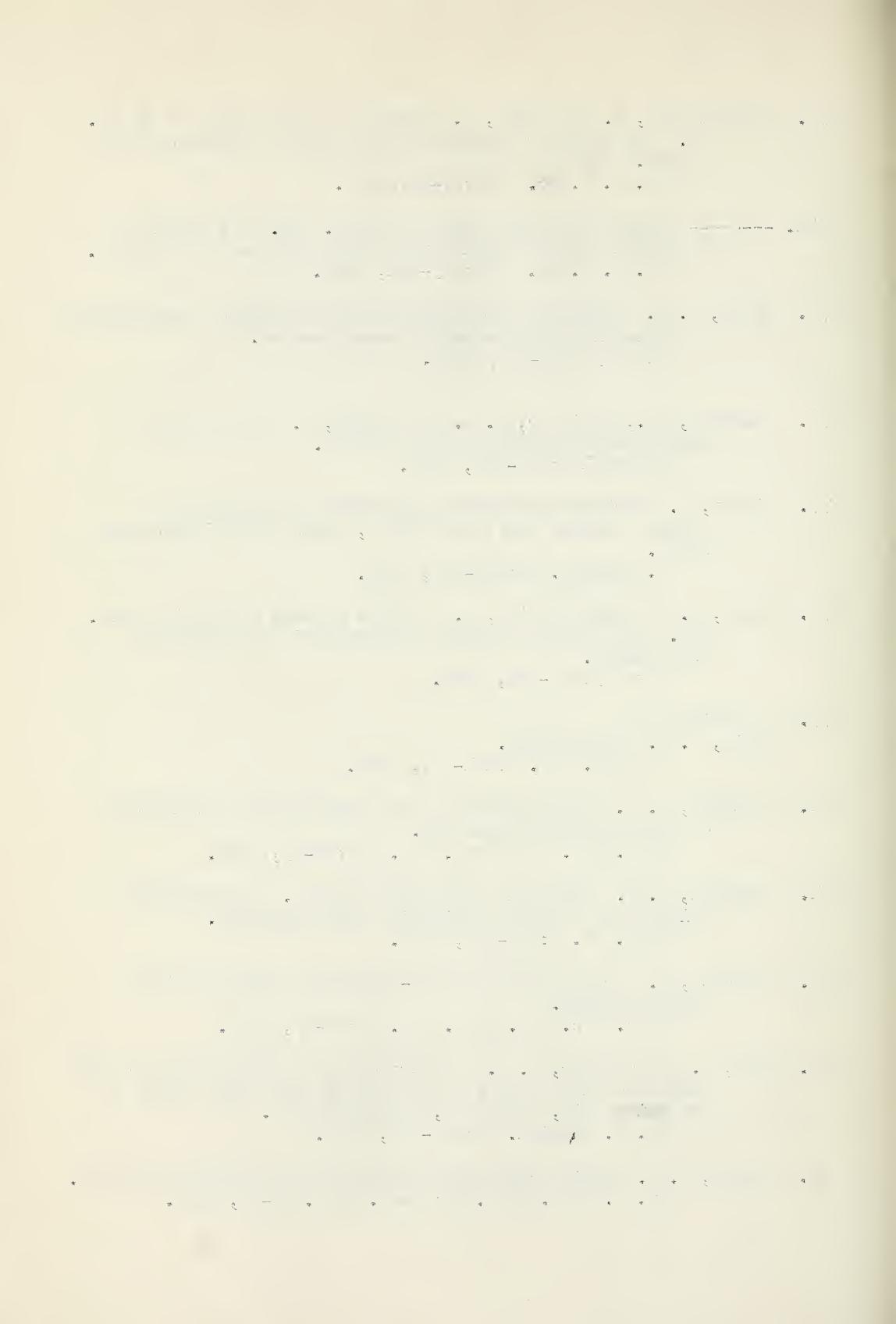
TABLES OF DATA FROM MARROW CULTURES

Culture	Time (hrs.)	Total Cells Conc.	%	Nucleated Cells Conc.	%	Non-nucleated Cells Conc.	%
42 A	0	950	100	675	100	275	100
	4	3500	368	600	89	2900	1055
B	0	1200	100	600	100	600	100
	4	2950	517	750	125	2200	367
C	0	13900	100	1475	100	12425	100
	4	13650	98	1325	90	12325	99
D	0	13250	100	1650	100	11600	100
	4	13400	101	1300	79	12100	104
43 A	0	20850	100	2450	100	18400	100
	3	14000	67	2425	99	11575	63
	6	16500	79	2150	88	14350	78
B	0	19500	100	2350	100	17250	100
	3	19000	97	2425	103	16575	96
	6	21500	110	2500	106	19000	110
C	0	5300	100	1500	100	3800	100
	3	6000	113	975	65	5025	132
	6	4800	91	750	50	4040	107
D	0	5300	100	1500	100	3800	100
	3	6050	114	850	57	5200	137
	6	5450	103	1150	77	4300	113
44 A	0	6300	100	2950	100	3350	100
	3	5650	90	2250	76	3400	101
	6	5050	80	1725	58	3325	99
B	0	5950	100	3225	100	2725	100
	3	6100	103	2125	66	3975	146
	6	5400	91	1550	48	3850	141
C	0	2800	100	2075	100	725	100
	3	2500	89	1450	70	1050	145
	6	3650	130	1450	70	2200	303
D	0	2600	100	2400	100	200	100
	3	3300	126	1775	74	1525	763
	6	3250	125	1325	55	1925	962

Culture	Time (Hrs.)	Total Cells		Nucleated Cells		Non-Nucleated Cells	
		Conc.	%	Conc.	%	Conc.	%
46 A	0	2800	100	2325	100	475	100
	3	3650	130	1450	62	2200	463
	6	3350	120	1000	43	2350	495
B	0	3650	100	2050	100	1600	100
	3	4000	110	1575	77	2425	152
	6	5050	138	1250	61	3800	238
C	0	2700	100	2100	100	600	100
	3	3600	133	1450	69	2150	358
	6	3450	128	1075	51	2375	396
D	0	4050	100	1750	100	2300	100
	3	4050	100	1500	86	2550	111
	6	4300	106	1325	76	2975	129
47 A	0	4750	100	3425	100	1325	100
	4	2850	60	1625	47	1225	92
B	0	4350	100	3450	100	900	100
	4	3450	79	1750	51	1700	189
C	0	4350	100	3500	100	850	100
	4	4950	114	2125	61	2825	332
D	0	4050	100	3300	100	750	100
	4	2900	72	1375	42	1525	203
63 A	0	9550	100	6425	100	3125	100
	4	8350	87	2250	35	6100	195
B	0	9600	100	5525	100	4075	100
	4	9300	97	2350	43	6950	171
C	0	6850	100	3625	100	3225	100
	4	11600	271	1325	37	10275	319
D	0	6650	100	3175	100	3475	100
	4	6400	96	1200	38	5200	150

Culture	Time (Hrs.)	Total Cells		Nucleated Cells		Non-Nucleated Cells	
		Conc.	%	Conc.	%	Conc.	%
65 A	0	17850	100	4425	100	13425	100
	4	17500	98	2600	59	14900	111
B	0	17400	100	4850	100	12550	100
	4	18450	106	2475	51	15975	127
C	0	18450	100	4350	100	14100	100
	4	16600	90	2925	67	13675	97
D	0	16550	100	4600	100	11950	100
	4	16700	101	2975	65	13725	115
70 A	0	28700	100	15350	100	13450	100
	4	33750	118	7700	50	26050	194
B	0	28900	100	15550	100	13350	100
	4	21800	75	5700	37	16100	121
C	0	27450	100	14100	100	13350	100
	4	21100	77	5950	42	15150	113
D	0	22450	100	10950	100	11500	100
	4	23550	105	6900	63	16650	145
71 A	0	12550	100	5550	100	7000	100
	4	13375	107	5550	100	7825	111
B	0	9350	100	3650	100	5700	100
	4	11450	122	4100	112	7350	129
C	0	10250	100	4475	100	5775	100
	4	10400	101	3775	84	6625	115
D	0	10650	100	4700	100	5950	100
	4	12255	115	4275	91	7975	134
72 A	0	15375	100	10175	100	5200	100
	4	7175	47	2075	20	5100	98
B	0	15125	100	9925	100	5200	100
	4	9600	63	3500	35	6100	117

Culture	Time (Hrs.)	Total Cells		Nucleated Cells		Non-Nucleated Cells	
		Conc.	%	Conc.	%	Conc.	%
73 A	0	8300	100	5950	100	2350	100
	4	4650	56	1900	32	2750	117
B	0	8200	100	5800	100	2400	100
	4	5250	64	2350	41	2900	121
C	0	8300	100	5950	100	2350	100
	4	5300	63	2300	39	2900	123
D	0	7750	100	5650	100	2100	100
	4						
74 A	0	11200	100	7450	100	3750	100
	4	8700	78	5450	73	3250	87
B	0	9650	100	6850	100	2800	100
	4	7650	79	4800	70	2850	102
C	0	10450	100	7400	100	3050	100
	4	7450	71	4450	60	3000	98
D	0	10700	100	7650	100	3050	100
	4	8350	78	5050	66	3300	108
75 A	0	3500	100	3100	100	400	100
	4	2300	66	1450	47	850	213
B	0	3350	100	2900	100	450	100
	4	2100	63	1500	52	600	134
76 A	0	1825	100	1275	100	550	100
	4	1400	77	725	57	675	123
B	0	1625	100	1050	100	575	100
	4	1275	78	650	62	625	109
C	0	1925	100	1250	100	675	100
	4	1200	62	650	52	550	81
D	0	1800	100	1225	100	575	100
	4	1175	65	650	53	525	91

Culture	Time (Hrs.)	Total Cells		Nucleated Cells		Non-Nucleated Cells	
		Conc.	%	Conc.	%	Conc.	%
77 A	0	17250	100	13250	100	4000	100
	4	9100	53	5200	39	3900	98
B	0	13750	100	10550	100	3200	100
	4	10050	73	5900	56	4150	130
C	0	15250	100	12350	100	2900	100
	4	14500	95	11000	89	3500	121
D	0	14900	100	12050	100	2850	100
	4	15600	105	11650	97	3950	139
78 A	0	19850	100	13600	100	6250	100
	4	12250	62	5450	40	6800	109
B	0	20150	100	14600	100	5550	100
	4	12300	61	5450	37	6850	123
C	0	18950	100	12750	100	6200	100
	4	17400	92	10100	79	7300	118
D	0	16850	100	11500	100	5350	100
	4	11750	70	9950	87	6800	127
79 A	0	18950	100	8250	100	10700	100
	4	16900	89	7400	90	9500	89
B	0	17750	100	9450	100	8300	100
	41	16250	92	7800	83	8450	102
C	0	17800	100	8100	100	9700	100
	4	16700	94	7450	92	9250	95
D	0	15850	100	7300	100	8550	100
	4	17400	110	8350	114	9050	105
E	0	17900	100	9550	100	8350	100
	4	16900	94	7550	79	9350	112
F	0	18950	100	9750	100	9200	100
	4	13650	72	5150	53	8500	92

Culture	Time (Hrs.)	Total Cells		Nucleated Cells		Non-Nucleated Cells	
		Conc.	%	Conc.	%	Conc.	%
80 B	0	36050	100	13700	100	22350	100
	4	36550	101	10550	77	26000	116
D	0	39050	100	14750	100	24300	100
	4	31400	80	7850	53	23550	97
A	0	30600	100	8600	100	20000	100
	4	29960	98	8000	93	21900	106
C	0	30100	100	7600	100	22500	100
	4	32000	106	8200	108	23800	106
81 B	0	22100	100	11300	100	10800	100
	4	21600	98	8900	79	12700	118
C	0	22800	100	11100	100	11700	100
	4	24000	105	11400	103	12600	108
D	0	20500	100	8800	100	11700	100
	4	23700	116	10500	119	13200	113
E	0	20100	100	8900	100	11200	100
	4	21800	108	9300	104	12500	112
F	0	21600	100	9200	100	12400	100
	4	21200	98	9400	102	11800	95
A	0	20100	100	7600	100	12500	100
	4	22700	113	9500	125	13200	106
82 B	0	10100	100	7500	100	2600	100
	4	13000	129	10100	135	2900	112
E	0	9700	100	7700	100	2000	100
	4	12200	126	9200	119	3000	150
C	0	13300	100	10400	100	2900	100
	4	13800	104	10400	100	3400	117
F	0	13600	100	10400	100	3200	100
	4	14700	108	11600	112	3100	97

Culture	Time (Hrs.)	Total Cells		Nucleated Cells		Non-Nucleated Cells	
		Conc.	%	Conc.	%	Conc.	%
83 A	0	41400	100	7900	100	33500	100
	4	40600	98	8900	113	31700	95
D	0	45900	100	11600	100	34300	100
	4	43900	96	10200	88	33700	98
B	0	42800	100	10900	100	31900	100
	4	44000	103	9400	86	34600	108
C	0	41600	100	9600	100	32000	100
	4	46200	111	10800	113	35400	111

ASSEMBLED RESULTS

I. CULTURES UNDER VARIOUS CONDITIONS

A. The Production of Erythrocytes

<u>1</u>. Balanced Salt Solution and Serum Medium – traumatic preparation.

Culture	Red Cell Conc. Change	Av. Nuc. Cell Conc.	Red Cell Conc. Change / Av. Nuc. Cell Conc.
42 C	– 100	1400	– 0.07
42 D	+ 500	1475	+ 0.34
47 A	– 100	2525	– 0.04
47 D	+ 775	2338	+ 0.33
			Average = + 0.14

<u>2</u>. Balanced Salt Solution and Serum Medium – atraumatic preparation.

Culture	Red Cell Conc. Change	Av. Nuc. Cell Conc.	Red Cell Conc. Change / Av. Nuc. Cell Conc.
57 A	+ 750	375	+ 2.00
57 B	+1050	263	+ 3.99
59 A	+ 400	1050	+ 0.38
59 B	+ 450	950	+ 0.47
63 C	+7050	2475	+ 2.85
63 D	+1725	2188	+ 0.79
		Average = 1.75	

<u>3</u>. Native Serum Medium – atraumatic preparation.

Culture	Red Cell Conc. Change	Av. Nuc. Cell Conc.	Red Cell Conc. Change / Av. Nuc. Cell Conc.
65 A	+1475	3513	+ 0.42
65 B	+3425	3663	+ 0.94
65 C	– 425	3638	– 0.12
65 D	+1775	3788	+ 0.47
73 A	+ 400	3925	+ 0.10
73 B	+ 500	4075	+ 0.12
73 C	+ 550	4125	+ 0.13
74 A	– 500	6450	– 0.08
74 B	+ 50	5825	+ 0.01
75 A	+ 450	2275	+ 0.20
75 B	+ 150	2200	+ 0.07
76 A	+ 125	1000	+ 0.12
76 B	+ 50	850	+ 0.06
		Average = 0.19	

4. Heated Native Serum Medium - atraumatic preparation.

Culture	Red Cell Conc. Change	Av. Nuc. Cell Conc.	Red Cell Conc. Change / Av. Nuc. Cell Conc.
79 B	+ 150	8625	+ 0.02
80 B	+3650	12125	+ 0.30
81 B	+1900	10100	+ 0.19
			Average = 0.17

5. Native Plasma Medium - atraumatic preparation.

70 A	+11600	11525	+ 1.01
B	+ 2750	10625	+ 0.26
C	+ 1800	10025	+ 0.18
D	+ 5150	8925	+ 0.58
71 A	+ 825	5550	+ 0.15
B	+ 1650	3875	+ 0.43
C	+ 850	4125	+ 0.21
D	+ 2025	4488	+ 0.45
72 A	- 100	6125	- 0.02
B	+ 900	6713	+ 0.13
			Average = 0.34

6. Normal Human Serum Medium - atraumatic preparation.

77A	- 100	9225	- 0.01
B	+ 950	8225	+ 0.12
78A	+ 550	9525	+ 0.06
B	+ 1300	10025	+ 0.13
			Average = 0.07

B. The Survival of Nucleated Cells

1. Balanced Salt Solution and Serum Medium - traumatic preparation.

Culture	% Survival
42 C	90
42 D	79
47 A	47
47 D	42
	Average = 64.5%

2. Balanced Salt Solution and Serum Medium - atraumatic preparation.

Culture	% Survival
57 A	58
57 B	50
59 A	50
59 B	33
63 C	37
63 D	38

Average = 44%

3. Native Serum Medium - atraumatic preparation.

65 A	59
B	51
C	67
D	65
73 A	32
B	41
C	39
74 A	73
B	70
75 A	47
B	52
76 A	57
B	62

Average = 55%

4. Heated Native Serum Medium - atraumatic preparation

79 B	83
80 B	77
81 B	79

Average = 80%

5. Native Plasma Medium - atraumatic preparation.

70 A	50
B	37
C	42
D	63

5. Native Plasma Medium - atraumatic preparation (Continued).

Culture	% Survival
71 A	100
B	112
C	84
D	91
72 A	20
B	35
	Average = 63%

6. Normal Human Serum Medium - atraumatic preparation.

77 A	39
B	56
78 A	40
B	37
	Average = 43%

II. THE EFFECT OF HEPARIN (Cell conc. in % at end of culture period.)

A. Native Serum Medium

Culture	Total	Control Nucleated	Red	Culture	Total	Experimental Nucleated	Red
74 A	78	73	87	74 C	71	60	98
74 B	79	70	102	74 D	78	66	108
76 A	77	57	123	76 C	62	52	81
76 B	78	62	109	76 D	65	53	91
Average	78	66	106	Average	69	58	94

B. Normal Human Serum Medium

	Control				Experimental		
Culture	Total	Nucleated	Red	Culture	Total	Nucleated	Red
77 A	53	39	98	77 C	95	89	121
77 B	73	56	130	77 D	105	97	139
78 A	62	40	109	78 C	92	79	118
78 B	61	37	123	78 D	70	87	127
Average	62	43	115		91	88	126

III. THE EFFECT OF AGITATING CULTURES (Cell conc. in % at end of culture period.)

	Agitated				Unagitated		
Culture	Total	Nucleated	Red	Culture	Total	Nucleated	Red
79 B	92	83	102	79 E	94	79	112
79 A	89	90	89	79 C	94	92	95
80 B	101	77	116	80 D	80	53	97
Average	94	83	102		89	75	101

IV. THE EFFECT OF LIVER EXTRACT (Cell conc. in % at end of culture period.)

	Liver extract 0.06μ/cc.				Control		
Culture	Total	Nucleated	Red	Culture	Total	Nucleated	Red
80 C	106	108	106	80 A	98	93	100
81 F	98	102	95	81 A	113	125	106
Average	102	105	101		106	109	103

	Liver extract 0.25μ/cc.				Control		
81 C	105	103	108	81 B	98	79	118

V.   THE EFFECT OF EMBRYO EXTRACT (Cell conc. in % at end of culture period.)

	Control				Experimental		
Culture	Total	Nucleated	Red	Culture	Total	Nucleated	Red
79 D	110	114	106	79 F	72	53	92

VI.   THE EFFECT OF COLCHICINE (Cell conc. in % at end of culture period.)

	Control				Experimental		
Culture	Total	Nucleated	Red	Culture	Total	Nucleated	Red
81 D	116	119	113	81 E	108	104	112

VII.   THE EFFECT OF FAT REMOVAL (Cell conc. in % at end of culture period.)

	Fat Removed				Fat not removed		
Culture	Total	Nucleated	Red	Culture	Total	Nucleated	Red
63 C	271	37	319	63 A	87	35	195
63 D	96	38	150	63 B	97	43	171
Average	184	38	235	Average	92	39	183

VIII.   THE USE OF PHYTOHEMAGGLUTININ (Cell conc. in % at end of culture period.)

A.   Four Hour Culture

	Experimental				Control		
Culture	Total	Nucleated	Red	Culture	Total	Nucleated	Red
42 A	368	89	1055	42 C	98	96	99
42 B	517	125	367	42 D	101	79	104
47 B	79	51	189	47 A	60	47	92
47 C	114	61	332	47 D	72	42	203
Average	270	82	486		83	65	125

B.   Six Hour Culture

43 C	91	50	107	43 A	79	88	78
43 D	103	77	113	43 B	110	106	110
44 D	130	70	303	44 A	80	58	99
44 E	125	55	962	44 B	91	48	141
46 A	120	43	495	46 B	138	61	238
46 C	128	51	396	46 D	106	76	129
Average	116	58	396		101	73	133

IX.   STORAGE OF MARROW CELLS BY REFRIGERATION

A.   The Effect on Cells of Storage at 4° C.

Series I

	0 Hrs.				24 Hrs.				48 Hrs.		
Culture	Total	Nuc.	Red	Culture	Total	Nuc.	Red	Culture	Total	Nuc.	Red
79 $\frac{B}{E}$	100	100	100	79 $\frac{D}{F}$	98	90	107	79 $\frac{A}{C}$	103	86	123
81 $\frac{B}{C}$	100	100	100	81 $\frac{D}{E}$	90	79	102	81 $\frac{A}{F}$	93	75	111
Average	100	100	100		94	85	105		98	81	117

Series II

Culture	O Hrs.			Culture	24 Hrs.		
	Total	Nucleated	Red		Total	Nucleated	Red
82 C F	100	100	100	82 B E	74	73	75
83 A D	100	100	100	83 B C	91	105	94
80 B D	100	100	100	80 A C	81	57	95
Average	100	100	100		84	78	88

B. The Ability of Stored Cells to Grow

Series I

Culture	O Hrs.			Culture	24 Hrs.			Culture	48 Hrs.		
	Total	Nuc.	Red		Total	Nuc.	Red		Total	Nuc.	Red
79 B	92	83	102	79 D	110	114	106	79 A	89	90	89
81 B	98	79	118	81 D	116	119	113	81 A	113	125	106
Average	95	81	110		113	117	110		101	108	98

Series II

Culture	Total	Nuc.	Red	Culture	Total	Nuc.	Red
80 B	101	77	116	80 A	98	93	100
82 C	104	100	117	82 B	129	135	112
83 A	98	113	95	83 B	103	86	108
Average	101	97	109		111	109	108

APPENDIX III

PHYTOHEMAGGLUTININ — PRELIMINARY INVESTIGATION

(Condensed from "Hemagglutination with Bean Extracts," a paper
presented to the Collip Research Club in October 1952 by the author.)

Bean extract was prepared following Osgood's method.
Two hundred grams of navy beans was soaked in a litre of 0.85% NaCl
for 24 hours. The beans and saline were then macerated in a Waring
Blender for half an hour and the resultant "cold bean soup" was
allowed to stand for 3 hours with frequent mixing. After centrifuging,
10 grams of "filter aid" were added to the supernatant which was then
filtered through a No. 30 Whatman paper in a Buchner funnel with
suction. The filtrate was brought to a pH of 7.4 with 20% NaOH and
filtered as before. For sterile work the preparation was filtered
through 5 micron Seitz discs.

Three weeks after its preparation the first extract
was found to be inactive. Bacteriological studies indicated the
presence of contamination. Because the 5 micron Seitz discs failed
to retain many bacteria and finer porosity do not allow filtration,
Seitz filtration was not used in subsequent preparations. To provide
some protection against bacterial contamination, penicillin was added
producing a concentration of 500 units per ml. of extract.

The preservation of bean extract for long periods
without bacterial contamination and inactivation remained a problem.
At the suggestion of Dr. W. C. Stewart a little bean extract in a

vial was frozen. Two days later after thawing, no loss of agglutin-
ating power could be detected. Subsequent extracts were therefore
kept in the frozen state without detectable loss of power for 6
weeks at least.

AGGLUTININS ON PERIPHERAL BLOOD

Four ml. of whole peripheral blood were placed in
each of 2 tubes and initial cell counts done. 0.05 ml. bean extract
was added to one and 0.05 ml. saline to the other. Both were spun
at 500 r.p.m. for 90 seconds. Erythrocytes were thrown down only in
the tube containing bean extract.

Table 1. Effect of Bean Extract on 4 ml. of Peripheral Blood

	Nucleate Counts	Non-Nuc. Counts	Ratio N/N-N
Blood + 0.05 ml. saline	5,600	5,300,000	1:964
Blood + 0.05 ml. bean	3,100	80,000	1:26
% Survival	55	1.3	

To demonstrate the difference between cells
agglutinated by bean extract and cells packed by centrifuging at
high speed, 10 ml. of red cell suspension was placed in each of
2 centrifuge tubes. One was spun at 3,000 r.p.m. for 30 minutes.

To the other 0.05 ml. of bean extract was added followed by centri-
fuging at 500 r.p.m. for 90 seconds. The tubes then presented a
similar appearance; the cells packed in the bottom with clear
supernatant.

Both tubes were simultaneously inverted several
times. The cells in the tube to which no bean extract had been
added quickly became resuspended. In the other tube large clumps
were visible which quickly settled out.

AGGLUTININS ON MARROW CELLS

The effect of bean extract on marrow cells was
next studied. After suspending rabbit marrow cells in an isotonic
saline medium, both total and nucleated cell counts were done. The
suspension was divided into samples of 5 ml. each. Retaining the
first tube as a control, extract was added to each of the others to
produce concentrations of bean extract of 0.20, 0.10, and 0.05 ml.
per cent. Following slow centrifuging, total and nucleated counts
were repeated. There was a marked reduction of erythrocytes and
only slight reduction of nucleates. The ratio of nucleates to reds
was increased.

Table 2. Effect of Bean Extract on 4 ml. of Marrow Suspension

Conc. of Bean Ext.	Nucleate Counts	Non-Nuc. Counts	Ratio N/N-N
0	4,900	23,600	1:4.8
0.20 ml.%	3,200	150	1:0.04
0.10 ml.%	4,100	5,400	1:1.3
0.05 ml.%	3,850	22,400	1:5.5

During the preparation of rabbit bone marrow for 5 separate cultures initial cell counts were done and the ratio of nucleated cells to red cells was determined. Then the marrow suspension was divided into two parts. An attempt at the removal of red cells was made by mechanical means in one portion. This consisted of placing the marrow cells in a Wintrobe tube and spinning at high speed, followed by removal of the nucleated layer, resuspension in saline, and cell counts.

The other half of the marrow was processed with bean extract. Since it is difficult to determine reliably, under the variable conditions present, the optimum amount of bean to be used, the procedure was adopted of adding 0.1 ml. of the extract to 50 ml. of marrow suspension, centrifuging for 90 seconds at 500 r.p.m., and doing cell counts on the supernatant. If an adequate removal of the erythrocytes had not been affected another 0.1 ml. of extract was added and the procedure repeated.

The following table compares mechanical removal of red cells from marrow with their precipitation by bean extract.

Table 3. Effect of Mechanical Separation and Bean Extract on Ratio of Nucleated to Non-Nucleated Cells.

Rabbit	Initial Ratio	Mechanical Separation	Bean Extract
42	1/10	1/7.7	1/0.59
43	1/3.3	1/7.7	1/2.5
44	1/1.6	1/1.1	1/0.13
45	1/3.3	1/4.3	1/0.38
46	1/1.0	1/1.0	1/0.24
Average	1/3.8	1/4.3	1/0.77

The mechanical separation does not change the ratio of nucleates to erythrocytes. The bean extract increased the ratio of nucleates to red cells.

HISTOLOGICAL STUDIES

A photomicrograph of a peripheral blood smear showed the large initial red cell population. After the addition of bean extract and spinning, a smear of the supernatant demonstrated a large decrease of the red cells in relation to the nucleated forms. A larger dose of bean extract was added, and after the usual slow spin, the supernatant was smeared, and showed very few erythrocytes though the nucleates were still fairly numerous. However, the percentage of polymorphs had decreas ed considerably.

A similar study was done with rabbit bone marrow. The marrow was treated with bean extract, and spun for 90 seconds at 500 r.p.m. A smear was made of the supernatant. There was a decrease in the number of red cells relative to the nucleates. Upon the addition of bean extract to marrow it was noticed that there was a loss of nucleated cells up to 50%. It was thought that the nucleates were being trapped in aggregates of red cell.

CONCENTRATION OF BEAN EXTRACT

An attempt was made to find the minimum effective concentration of bean extract required for agglutination. It seemed possible that the number of red cells agglutinated by 1 ml. of bean extract could be determined. However, it appears that besides the absolute number of red cells present, the concentration or density of cells, presence or absence of plasma, size and shape of tube, angle of tube during spinning, and variations between different extract preparations, all affect the degree of agglutination.

TEMPERATURE

The effect of temperature on the rate of agglutination first came to our attention while three attempts to demonstrate the action of the extract had ended in failure, when it was noticed that the cell suspension used, just removed from the refrigerator, was very

cold. Warming of the cell suspension resulted in a successful agglutination. Subsequent studies indicated that as the temperature is decreased from 37° C. to 0° C., the agglutinating activity of the bean extract becomes progressively less.